SUPPLEMENTARY INFORMATION

A Non–Perturbative Molecular Grafting Strategy for Stable and Potent Therapeutic Peptide Ligands

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Supplementary Figures

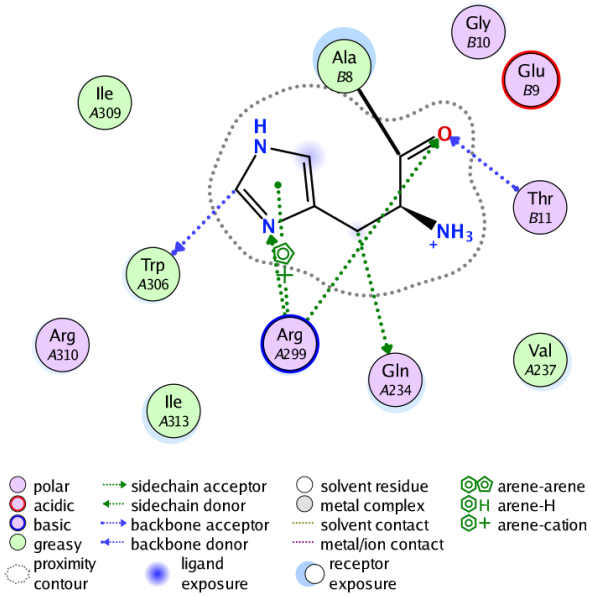
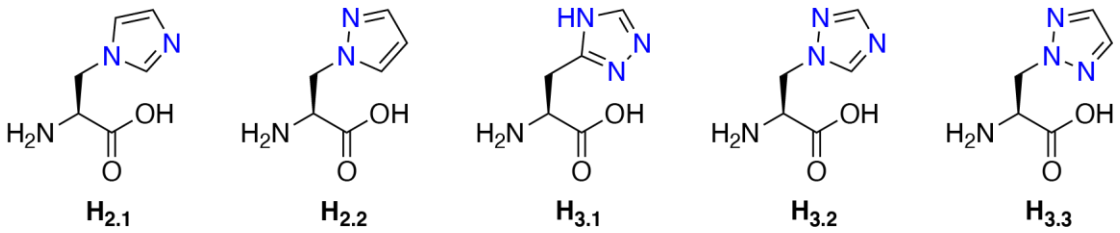


Figure S1A | Two-dimensional diagram generated using Molecular Operating Environment v2019.01 (Chemical Computing Group) of the *N*-terminal histidine in GLP1 (structure shown) and neighboring amino acids (A chain is receptor and B chain is the ligand). All interactions between the ligand and nearby amino acids are shown with the identity of each interaction described in the legend.



	EC_{50} (pM)	pEC_{50} ± SEM (pM)	*n*
GLP1	1.6	11.79 ± 0.05	6
$H_{2.1}$-GLP1(8-36)	640	9.19 ± 0.07	6
$H_{2.2}$-GLP1(8-36)	3.4	11.47 ± 0.05	3
$H_{3.1}$-GLP1(8-36)	760	9.12 ± 0.11	5
$H_{3.2}$-GLP1(8-36)	548	9.26 ± 0.05	7
$H_{3.3}$-GLP1(8-36)	7.5	11.12 ± 0.06	3

Figure S1B | His7 of GLP1 was mutated to unnatural amino acids ($H_{2.1}$, $H_{2.2}$, $H_{3.1}$, $H_{3.2}$, or $H_{3.3}$) to afford GLP1 analogues named $H_{2.1}$-GLP1(8-36), $H_{2.2}$-GLP1(8-36), $H_{3.1}$-GLP1(8-36), $H_{3.2}$-GLP1(8-36), and $H_{3.3}$-GLP1(8-36). HEK293 cells over-expressing GLP1R and CRE_{6x}-luciferase reporter were treated with GLP1 analogues to test potency and efficacy at GLP1. Production of luciferase represents activation of GLP1R in a cAMP-dependent manner. EC_{50} represents the concentration of the peptide required for half-maximal activation of GLP1R and pEC_{50} = –log(EC_{50}) ± standard error of the mean (SEM) for number of independent experiments (n). Results shown in the table illustrate the importance of number and location of nitrogens within the side chain of the first amino acid of GLP1, His7.

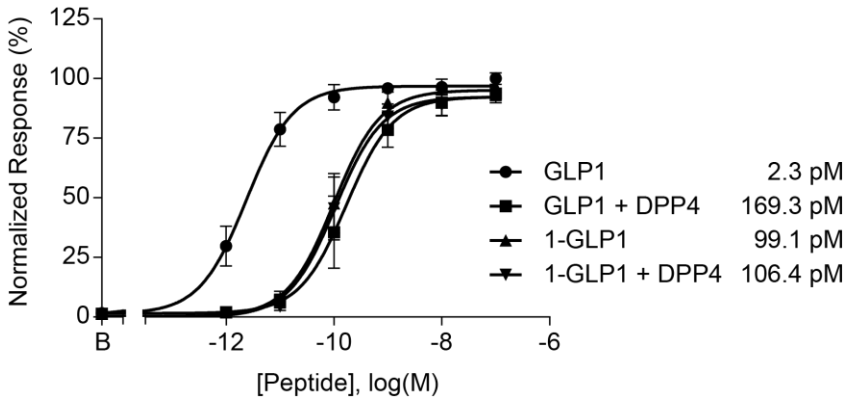


Figure S2 | Concentration response curve of peptides GLP1 or **1-GLP1** incubated with DPP4 or vehicle overnight with HEK-293 cells overexpressing GLP1R and luciferase reporter. The EC_{50} corresponding to each peptide is listed in the legend. Data represent the mean of one experiment conducted in triplicate ± SEM.

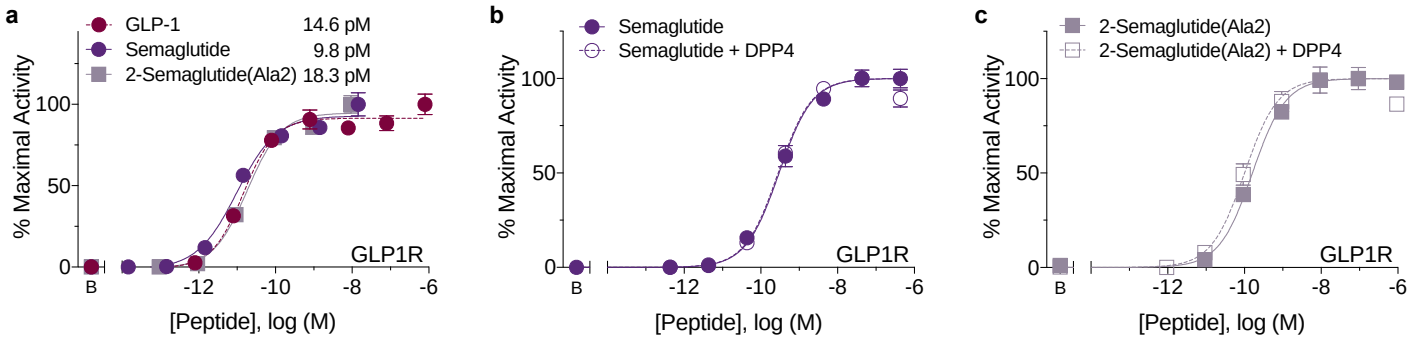
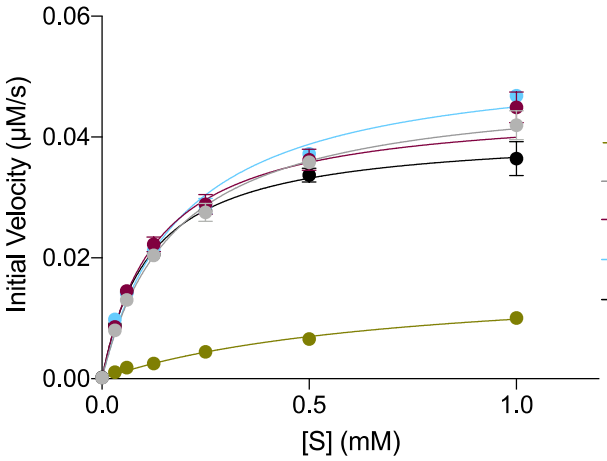


Figure S3 | Representative concentration response curve of (**a**) GLP1, semaglutide, and **2-semaglutide(Ala2)**. Peptides (**b**) Semaglutide or (**c**) **2-Semaglutide(Ala2**) were incubated overnight with vehicle or DPP4 at 37 °C. All peptides and enzyme mixtures were tested on HEK293 cells over-expressing GLP1R and luciferase reporter. Luciferase production corresponds directly to activation of GLP1R via a cAMP dependent pathway, normalized to 100% maximal activity. Data represent the mean of one experiment conducted in triplicate ± SEM.



	K_m ± SEM (µM)	V_{max} (mM/s)
Linagliptin (10 nM)	704 ± 100	16.8 ± 2
GLP1 (2 µM)	174 ± 10	48.6 ± 1
2-GLP1 (2 µM)	131 ± 8	45.2 ± 1
12-GLP1 (2 µM)	190 ± 30	53.6 ± 3
Vehicle	116 ± 5	40.9 ± 1

Figure S4 | Michaelis-Menten kinetics of DPP4 (preincubated for 30 minutes at room temperature with linagliptin, GLP1, 2-GLP1, 12-GLP1, or vehicle was determined by the hydrolysis of the substrate GlyPro-pNA in TRIS buffer (pH 8.0). The initial velocity of product formation was measured by linear fit of pNA production (absorbance at 405 nm) within the first 5 minutes. Each data point represents the mean ± standard error of the mean (SEM) of three independent experiments. Data were fit using the equation: $V_0 = V_{max}[S]_0/(K_m+[S]_0)$ (GraphPad Prism, v8). Calculated K_m and V_{max} are shown in the table to the right of the legend.

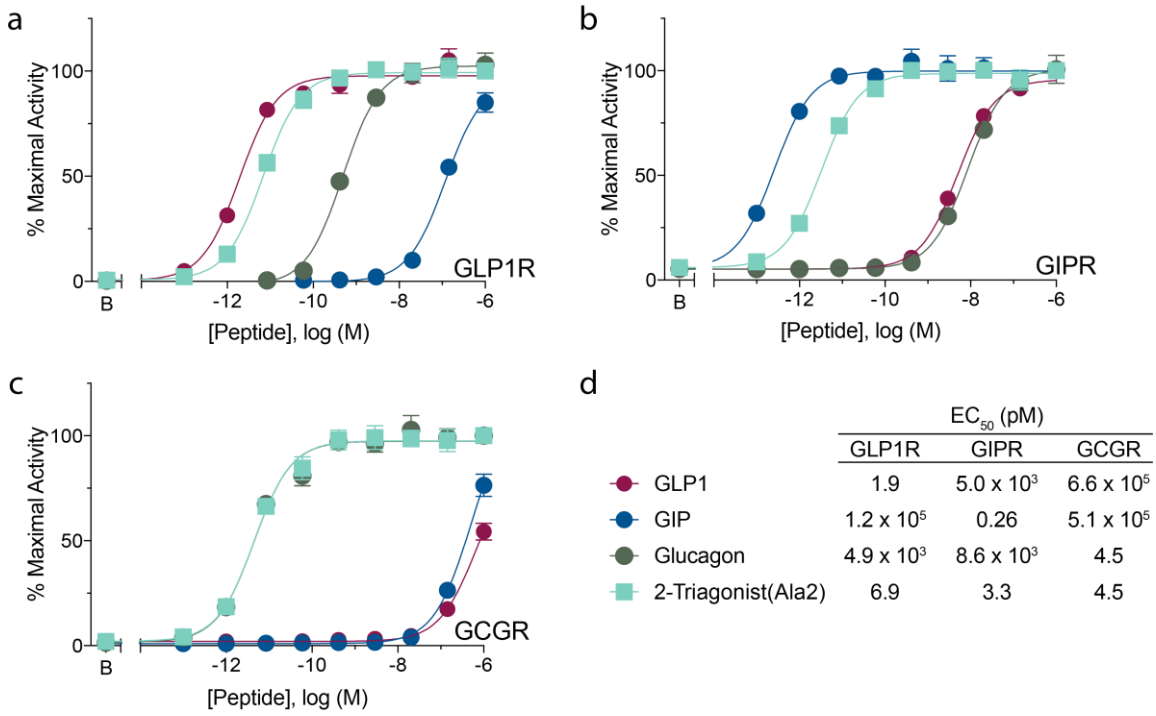


Figure S5 | Representative concentration response curves of unmodified peptides (GLP1, GIP, and GCG) and **2-triagonist(Ala2)** incubated with HEK293 cells over-expressing the luciferase reporter and target secretin receptor: (**a**) GLP1R, (**b**) GIPR, or (**c**) GCGR. Luciferase production corresponds directly to activation of the cognate GPCR *via* a cAMP dependent pathway, normalized to 100% maximal activity. Panel (**d**) shows legend along with corresponding EC_{50}s (from panels a-c) which were calculated from the concentration of peptide required to reach half maximal activity of target GPCR. Data represent the mean of one experiment conducted in sextuplicate ± SEM.

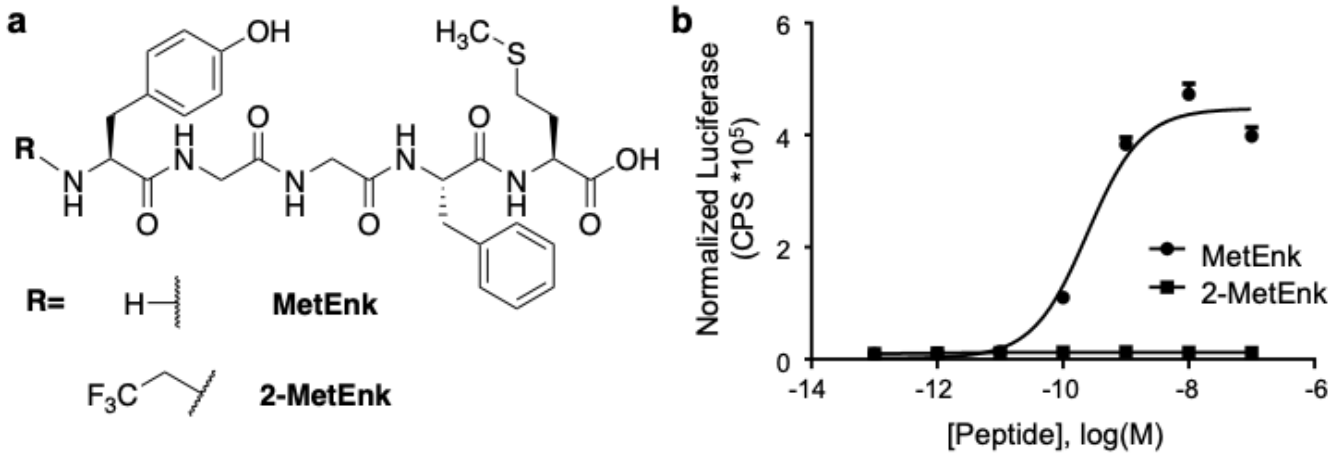


Figure S6 | (**a**) Structure of opioid peptide MetEnk and trifluoroethyl modified **2-MetEnk**. (**b**) Representative concentration response curves of MetEnk and **2-MetEnk** dosed in 96-well plate containing HEK293 cells overexpressing μ-opioid receptor and luciferase reporter. Luciferase production corresponds directly to activation of μ-opioid receptor. Data represent the mean of one experiment conducted in triplicate ± SEM.

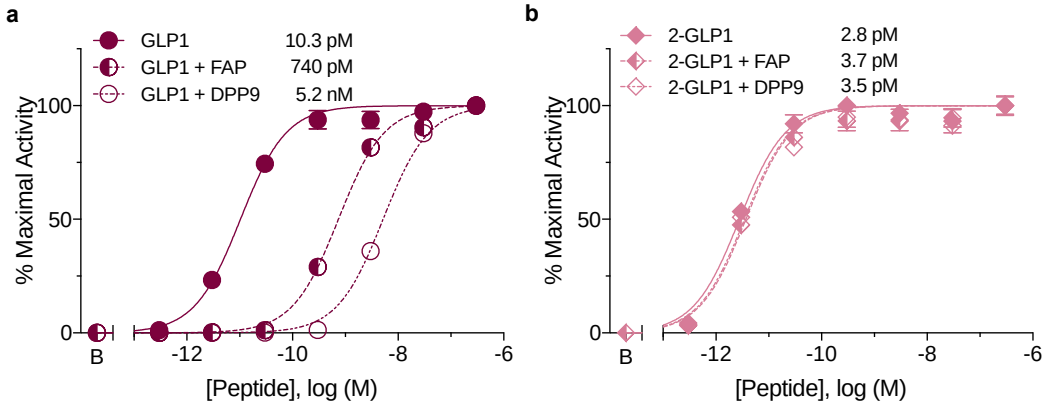


Figure S7 | Representative concentration response curves of (**a**) GLP1 and (**b**) **2-GLP1** preincubated overnight with vehicle, fibroblast activation protein (FAP), or dipeptidyl peptidase-9 (DPP9). with HEK293 cells overexpressing luciferase reporter and GLP1R. Data represents the mean of one experiment conducted in triplicate ± SEM with corresponding EC_{50} shown to the right of the legend. Luciferase production relates directly to activation of GLP1R *via* a cAMP dependent pathway, normalized to 100% maximal activity.

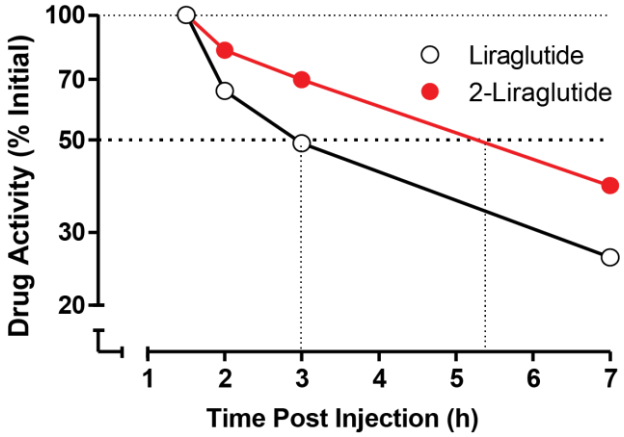


Figure S8 | *In vivo* protraction (serum stability) after i.v. administration of liraglutide (5 nmol/kg) or **2-liraglutide** (5 nmol/kg) in rats (n=1). After 7 hours, the *N*-trifluoroethylated peptide **2-liraglutide** showed enhanced serum stability with a half-life of 5.5 h compared to liraglutide ($t_{1/2}$= 3 h).

Safety Statement

Experiments were performed wearing a lab coat, gloves, and safety glasses. Tissue culture and handling of biosafety level 2 materials (HEK293 cells) was performed in a certified biosafety cabinet according to Tufts University health and safety standards. All chemical synthesis was performed in a fume hood. Extra care must be taken when working with trifluoroacetic anhydride and triflimide as both are extremely volatile and corrosive. Unreacted trifluoroacetic anhydride must be quenched prior to disposing in hazardous waste stream. HATU, HBTU, and amino acids were weighed out carefully wearing a mask to prevent inhalation and skin exposure as these reagents are known sensitizing agents.

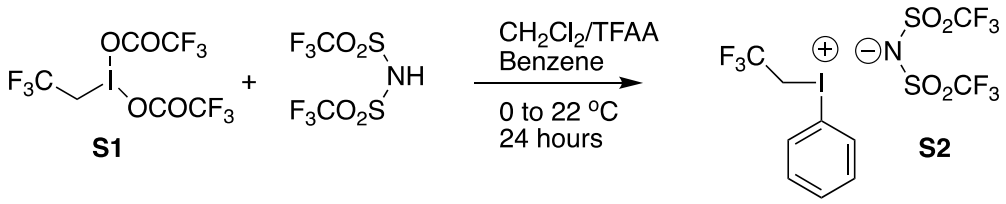
Materials and Methods

Synthesis of iodonium salt $(CF_3SO_2)_2NI(Ph)CH_2CF_3$ (S2)

S2 is a known compound[1]: we modified our published syntheses replacing fluorinated solvents with dichloromethane. For operational expedience, the following procedure was scaled to use one 25-gram bottle of commercial bis-trifluoromethylsulfonyl imide (triflimide). **Caution**: Both trifluoroacetic anhydride and triflimide are very corrosive and volatile: it is imperative to wear appropriate protection and work under an efficient fume hood.

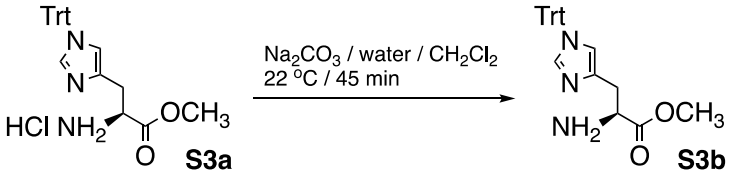
Intermediate **S1** was prepared as follows: A 500-mL round bottom flask with side arm was flushed with a slow stream of nitrogen, cooled in an ice-salt bath, and charged with trifluoroacetic anhydride (TFAA, 120 mL, 0.86 mol). After allowing to cool to –18 °C, 30% (w/w) hydrogen peroxide (10.18 g, 90 mmol) was added slowly dropwise with rapid stirring for 20 minutes. **Caution:** Exercise care to avoid splattering and overheating: the addition can be interrupted and resumed as needed.

After stirring 5 additional minutes, CF_3CH_2I (18.86 g, 90 mmol) was added rapidly. The ice/salt cooling was replaced with ice/water and the clear solution was stirred at 0–6 °C under static nitrogen for 30 minutes, then at ambient temperature, protected from light with aluminum foil, for 24 hours. The volatiles were rotary-evaporated and the resulting oil solidified on cooling. After one hour under oil-pump vacuum were obtained 36.1 g of $CF_3CH_2I(OCOCF_3)_2$ as a white crystalline solid (82.8 mmol, 92%).[2] $CF_3CH_2I(OCOCF_3)_2$ is unchanged after six months in a freezer. In moist air, it should be handled quickly because it is hygroscopic. **Caution:** unreacted trifluoroacetic anhydride must be quenched for safety and not disposed as such into the waste stream. On this reaction scale, we preloaded the receiving flask of the rotary evaporator with 200 mL of isopropanol and cooled the flask in dry ice; the resulting isopropanol/trifluoroacetic acid mixture was then disposed into acid waste.

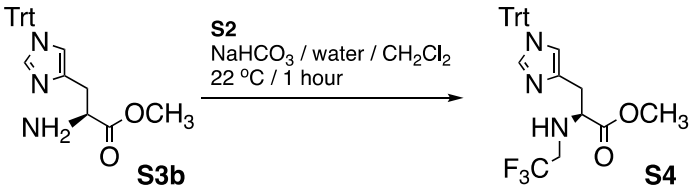


A 250-mL round bottom flask with side arm was flushed with a very slow stream of nitrogen, then using a wide-bore funnel, rapidly charged with all of the $CF_3CH_2I(OCOCF_3)_2$ **S1** above and triflimide (25 g, 89 mmol), using dry dichloromethane (50 mL in total) to flush all the solids into the flask. Trifluoroacetic anhydride (11 mL, 81 mmol) was added in one portion and the slurry was stirred under static nitrogen at ambient temperature for 30 min, protecting from light with aluminum foil, until it became a translucent solution. The flask was cooled with ice/water and briefly opened while benzene (9 mL, 100 mmol) was added in 2 minutes. After 10 minutes in ice, the reaction was continued at ambient temperature under static nitrogen for 24 hours. The volatiles were evaporated, and the brown residue held under oil-pump vacuum for 2 hours. Crushed ice was added causing a white precipitate to form. The precipitate was broken up and stirred rapidly with more ice and water. Sufficient sodium bicarbonate was added in small portions to obtain a neutral pH (litmus). This removed most of the residual trifluoroacetic acid and excess triflimide. The suspension was filtered by suction on a glass frit and air-dried to a gray powder, which was further vacuum-dried to a weight of 37.3 g. This powder was dissolved with gentle warming in dichloromethane (110 mL) and the solution was cooled in a freezer overnight. The resulting white needles were washed with 20 mL dichloromethane (also cooled in the freezer) and pumped to a constant weight of 31.45 g (55.5 mmol, 62% based on triflimide). Crystalline **S2** was divided into 2.0-gram (ca 3.5 mmol) portions to serve in the synthesis of CF_3CH_2-His(Trt)-OH **S5**, below, and of other *N*-trifluoroethyl amino acids. It is storable in a refrigerator for years and for at least days at room temperature, if protected from light.

Synthesis of CF_3CH_2-His(Trt)-OMe (S4):

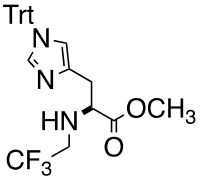


Commercial H-His(Trt)-OMe hydrochloride **S3a** (Chemimpex #06075) (1.795 g, 4 mmol) and sodium carbonate monohydrate (4.96 g, 40 mmol) were rapidly stirred in a mixture of water (50 mL) and dichloromethane (50 mL) for 45 min. The lower dichloromethane phase containing H-His(Trt)OMe **S3b** was separated into a 250 mL round bottom flask and the aqueous phase was discarded. **Note:** the dichloromethane phase may be slightly turbid, this does not affect the subsequent reaction.

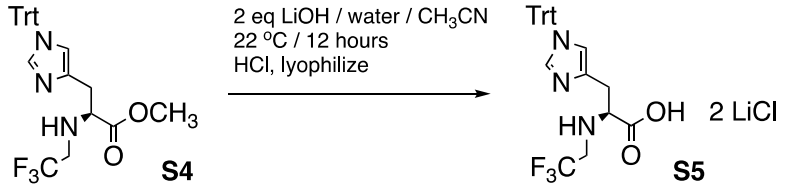


Into the solution of H-His(Trt)-OMe **S3b** at ambient temperature was added sodium bicarbonate (800 mg, 9.5 mmol) in water (30 mL). Under rapid stirring, iodonium salt **S2** (2.032 g, 3.58 mmol) was added in a single portion. The reaction was continued at ambient temperature for 1 hour. The organic phase was separated and dried over sodium sulfate, filtered and rotary-evaporated to a thick oil.

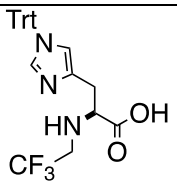
Crude CF_3CH_2-His(Trt)-OMe **S4** was purified by chromatography on an Isco CombiFlash apparatus with a solvent system of 55/45 ethyl acetate/hexanes (solvent A) and methanol (Solvent B), gradient 0 to 5% B in 5 minutes then constant 5% B. A 40-gram disposable column (Isco Redi-Sep 69-2203-340, normal phase silica gel) was used. Smaller columns may result in poor separation. The elution is in the order iodobenzene, unknown impurity (strongly UV absorbing), and desired product at ca. 2, 5, and 7 minutes. (TLC: R_f = 0.4 in hexanes/ ethyl acetate = 55/45 containing 5% methanol.) Separation of product from higher-R_f impurity was usually not baseline. Overlapping fractions in the chromatogram were checked by TLC and discarded if not one spot. The good fractions were rotary-evaporated to an oil. This was dissolved in 5-10 mL of 50/50 ether/hexane and rotary-evaporated to azeotrope residual ethyl acetate. The resulting white powder was held in oil-pump vacuum to constant weight. Ten replicate preparations gave 31-54% isolated yields of **S4**, average 41%, median 42%

S4	
^{1}H NMR	(500 MHz, CD_3CN) δ 2.82-2.83 (d, 2H, CH2), 3.06-3.15 (m, 1H, CH), 3.30-3.39 (m, 1H, CH), 3.57 (s, 3H), 3.63 (t, 1H, CH), 6.65 (s, 1H), 7.15-7.17 (m, 6H, ArH), 7.34 (d, 1H, ArH), 7.37-7.39 (m, 9H, ArH)
^{13}C NMR	(125 MHz, CD_3CN) δ 32.39, 49.42, 52.22, 62.13, 75.88, 120.19, 129.04, 130.46, 137.98, 139.02, 143.66, 174.75.
^{19}F NMR	(470 MHz, CD_3CN) δ -73.56 (t, J=9.8 Hz)
HRMS (ESI)	Calculated for $C_{28}H_{26}F_3N_3O_2$ 494.2050, found 494.2050

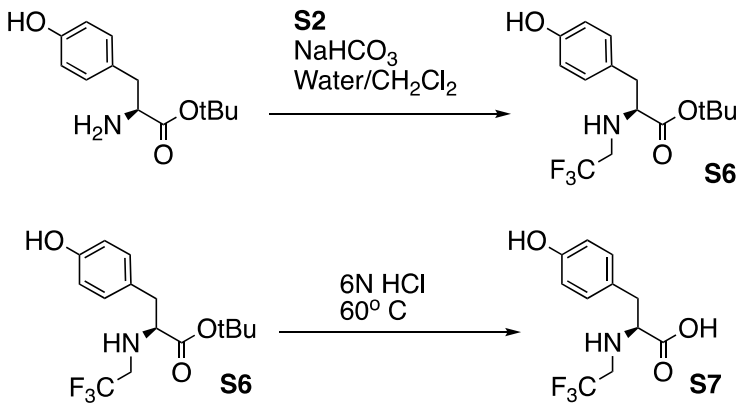
Synthesis of CF_3CH_2-His(Trt)-OH S5



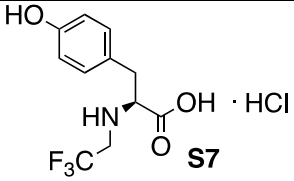
CF_3CH_2-His(Trt)-OMe **S4** described above 725 mg (1.47 mmol) was dissolved in 15 mL of acetonitrile. LiOH monohydrate (123 mg, 2.94 mmol) and 15 mL of water were added, and the mixture was stirred at ambient temperature overnight. Approximately 7 mL 1M aqueous HCl were then added until slightly acidic pH (litmus). Acetonitrile was rotary-evaporated at ambient temperature. The resulting slurry of product and lithium chloride was lyophilized to constant weight. The yield of dry product containing two equivalents LiCl (total mol wt. 564.28) was quantitative, and this was used in peptide synthesis directly. For NMR and HRMS analysis, CF_3CH_2-His(Trt)-OH · 2 LiCl (10 mg) in an Eppendorf vial was vortexed in DI water (1 mL) and centrifuged. This was repeated three times. The lyophilized white powder gave a clear solution in CD_3CN.

S5	
^{1}H NMR	(500 MHz, CD_3CN) 2.88-2.89 (d, 2H, CH2), 3.10–3.19 (m, 1H, CH), 3.31-3.40 (m, 1H, CH), 3.55 (t, 1H, CH), 6.78 (d, 1H), 7.17-7.19 (m, 6H, ArH), 7.34 (d, 1H, ArH), 7.37-7.39 (m, 9H, ArH), 7.52 (d, 1H)
^{13}C NMR	(125 MHz, CD_3CN) δ 31.10, 49.17-49.42, 61.59, 76.52, 120.55, 129.17, 130.48, 138.47, 143.20, 175.26.
^{19}F NMR	(470 MHz, CD_3CN) δ -73.14 (t, J=10 Hz)
HRMS (ESI)	Calculated for $C_{27}H_{24}F_3N_3O_2$ 480.1893, found 480.1914

Synthesis of CF_3CH_2-Tyr-OH S7

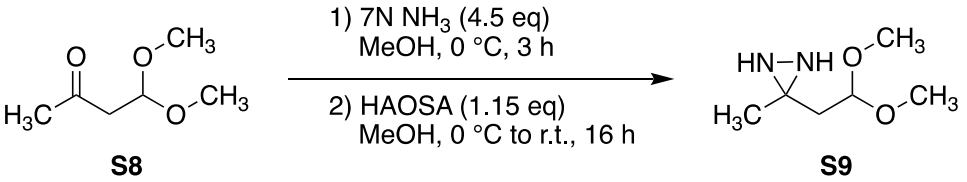


This is a slight variation of a known preparation[1]: commercial H-Tyr-OtBu (0.956 g, 3.5 mmol) was dissolved in dichloromethane (30 mL). (Note: unlike **S3a**, H-Tyr-OtBu is not a hydrochloride salt and no neutralization steps are needed). Sodium bicarbonate (800 mg, 9.5 mmol) dissolved in water (30 mL) was added. Under rapid stirring, iodonium salt **S2** (2.05 g, 3.61 mmol) was then added in one portion at room temperature. After one hour, the organic phase containing **S6** and the coproduct iodobenzene was separated and washed with water once. 6N HCl (30 mL) was added and the biphasic mixture was stirred and heated in a 60 °C bath for one hour. The aqueous phase was separated and concentrated to a small volume by rotary evaporation (Note: iodobenzene and tert-butanol co-evaporate with the constant-boiling 6N HCl) and then cooled at 5 °C overnight to precipitate **S7**. The supernatant (ca. 2.5 mL) was pipetted out and the solid was lyophilized yielding 719 mg (68%) of **S7** which is known to be an HCl salt.[1]

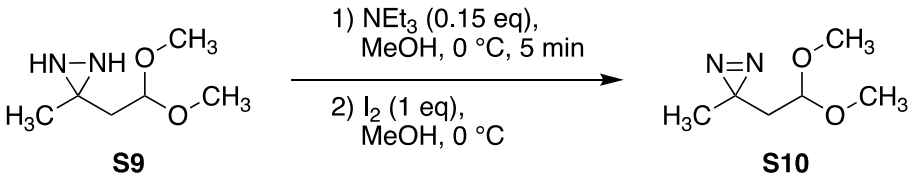
S7	
^{1}H NMR	(MeOH, 500 MHz) δ: 7.15 (d, 2H, 8.5 Hz), 6.79 (d, 2H, J 8.0 Hz), 4.32 (dd, 1H, J = 1.5 Hz, J = 6.00 Hz), 4.03 (td, 2H, J = 3.0 Hz, J = 9.0 Hz), 3.29 (d, 1H, J = 6.0 Hz), 3.19 (dd, 1H, J = 7.0 Hz, J = 7.5 Hz)
^{13}C NMR	(MeOH, 125 MHz) δ: 130.18, 115.34, 61.77, 46.04, 34.28
^{19}F NMR	(MeOH, 470 MHz) δ: 69.47 (t, J=10 Hz)
ESI	ESI+: calcd for [C11H12F3NO3 + H+] 264.09, found 264.09 [M + H+]

Synthesis of diazirine acetal S10

The following procedure combined a previously published protocol[3] to synthesize known compound **S10**, diazirine acetal.[4]

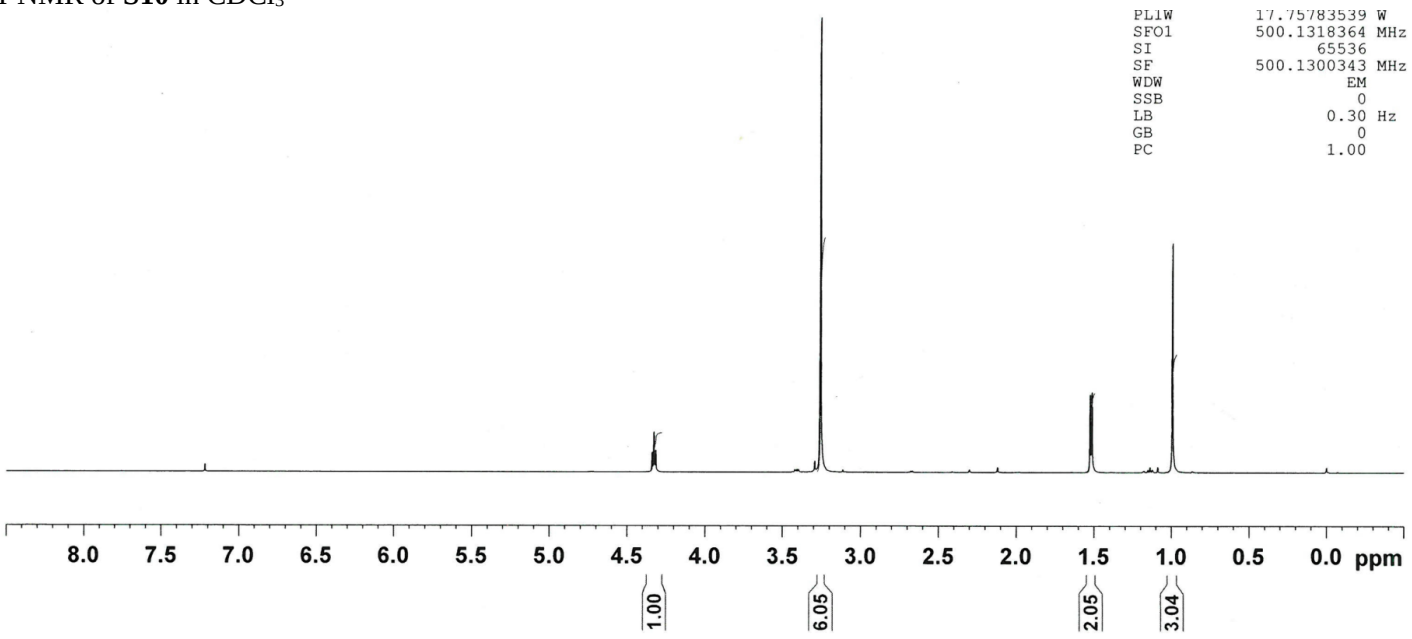


A 250 mL round bottom flask was charged with a stir bar and acetylacetaldehyde dimethyl acetal (**S8**, Alfa Aesar, 10 mmol). The solution was cooled to 0 °C and 10 mL of 7 N methanolic ammonia (Sigma-Aldrich) was added to the round bottom flask with a balloon to regulate pressure. The reaction was stirred at 0 °C for 3 hours. Hydroxylamine-*O*-sulfonic acid (HAOSA, 11.12 mmol, Sigma-Aldrich) was dissolved in 8.25 mL of methanol and slowing added this HAOSA/methanol mixture to the reaction flask at a steady rate of 1 drop per second. The reaction was allowed to stir for 16 h while the ice-water bath warmed to room temperature (note: formation of precipitate). The ammonia was removed by gentle bubbling of nitrogen through the suspension for 1 hour. The white precipitate was separated from the pale-yellow solution by vacuum filtration and the resulting liquid was concentrated *in vacuo* to yield crude diaziridine, **S9**, as a yellow/white residue.

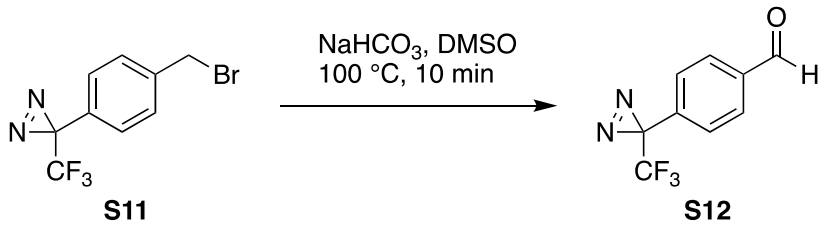


Crude diaziridine acetal **S9** was dissolved in MeOH (7 mL) and was placed in an ice-water bath and stirred for 5 minutes to cool in dark. Triethylamine (2 mL) was added and the solution was stirred for 5 minutes. Iodine was gradually added to the solution until the solution remained a persistent red-brown color for more than 3 minutes to oxidize diaziridine to diazarine **S10**. The crude reaction was extracted with 50 mL of ether and the organic layer was washed with 1 M aqueous HCl. The organic layer was further washed with 10% thiosulfate to reduce iodine to iodide which turned the organic layer from orange-red to colorless. Thiosulfate wash was repeated until the organic layer became colorless. The organic layer was extracted a final time with saturated NaCl, dried with sodium sulfate, and concentrated *in vacuo* to afford a yellow-orange liquid of diazirine acetal, **S10**, in 23% yield and stored at –20 °C.

^{1}H-NMR of **S10** in $CDCl_3$



Synthesis of S12



The procedure for the synthesis of the diazirine benzylaldehyde was followed as previously reported.[5] In summary, $NaHCO_3$ (16 mmol) was dissolved in 10 mL of DMSO and charged with benzyl bromide diazirine (1.2 mmol). The solution was heated to 100 °C, then cooled to room temperature after 10 min. The mixture was added to 100 mL of ice-water saturated with NaCl and the aqueous solution was extracted with diethylether (2 x 50 mL). The resulting organic layer was washed with saturated $NaHCO_{3(aq)}$ (1 x 25 mL) and brine (1 x 25 mL), dried with sodium sulfate, and concentrated *in vacuo*. The crude product was used directly for peptide alkyation on solid support. Analytical data correspond to those previosuly reported.

Peptide synthesis and purification. All peptides were synthesized by standard 9-fluorenylmethoxycarbonyl (Fmoc) chemistry on Rink amide, Wang, or MBHA solid supports (0.1 mmol). Each N^{α}-Fmoc amino acid (4 eq, 0.4 mM) activated with O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-phosphate (HATU, 3.6 eq) or 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HBTU, 3.6 eq) and N,N-diisopropylethylamine (DIEA, 8 eq) in dry dimethylformamide (DMF) for 2 minutes. The activated amino acid solution was added to the resin and bubbled with N_2(g) for 1 hour. After each protected amino acid coupling, N^{α}-Fmoc was deprotected with 20% (v/v) piperidine in DMF for 5 min followed by 15 min and washed (5x) with DMF. For *N*-terminally alkylated peptides, after Fmoc deprotection, the peptide resin was stirred with aldehyde (1M, 1 mL, 10 eq) in dry DMF to form an imine. After one hour, the DMF solution was drained and methanol/CH_2Cl_2 (1:3; v/v) was added and imine was reduced with the addition of $NaBH_4$ or $NaBH_3CN$ (25 mg). For lipidated peptides, the N^{ε}-amine of lysine was orthogonally protected with Alloc and deprotected with $Pd(PPh_3)_4$ (60 mg) and phenylsilane (110 mg) in CH_2Cl_2 (5 mL) for 4 hours in the dark.[6]

After peptide synthesis was completed, the resin was washed with CH_2Cl_2 (5x) and dried under reduced pressure overnight. Peptides were cleaved from the resin and sidechains were simultaneously deprotected with 2.5% (v/v) triisopropylsilane (TIPS), 2.5% (v/v) H_2O, and 95% (v/v) trifluoroacetic acid (TFA) for 90 minutes. The cleaved peptide was filtered from the resin, concentrated under reduced pressure, and precipitated with cold ether. The crude mixture was purified by reverse-phase high performance liquid chromatography (RP-HPLC) on a semi-prep C18 column (Vydac, 10 mm x 250 mm, 10 μm), 2.5 mL/min, with solvents A (99% (v/v) H_2O, 1% (v/v) acetonitrile, 0.1% (v/v) TFA) and B (10% (v/v) H_2O, 90% (v/v) acetonitrile, 0.07% (v/v) TFA). Purity of peptide were assessed on an analytical C18 column (Vydac, 4.6 mm x 250 mm, 5 μm), 1 mL/min, 20%-60%B over 20 minutes and all were >95% pure. Masses were measured by electrospray ionization mass spectrometry (ESI-MS, ThermoFisher) and/or MALDI-TOF-MS (Bruker).

Synthesis of 13-GLP1 and 14-GLP1. GLP1(7-36) was synthesized on Rink amide resin following standard Fmoc SPPS. The Fmoc protecting group was removed by 20% piperidine/DMF leaving a free amine on His7. The peptide on solid support was treated with 1 M (*R* or *S*)-Garner's aldehyde[7] in NMP to form an imine. After 1 hour, the solution was drained, and the resin was washed with CH_2Cl_2 three times. The imine was reduced with $NaBH_4$ (30 mg) in 25% Methanol/CH_2Cl_2 (4 mL). After 30 min, the solution was drained, and the resin was immediately washed with methanol to quench any remaining $NaBH_4$. The resin was dried as usual and cleaved in 95% TFA, 2.5% TIPS, and 2.5% H_2O to yield **13-GLP1** or **14-GLP1**.

Plasmid constructs. All plasmid constructs used in this study have been previously reported.[8] In summary, complementary DNAs encoding the following human receptors: GLP1R, GCGR, GLP2R, or ∂-receptor were obtained from the Missouri S&T cDNA Resource Center (www.cdna.org) and subcloned in pcDNA1.1.[9]

Cell culture. Human embryonic kidney (HEK) 293 cells (gift from Jefferson Lab, Tufts University) were grown in Dulbecco's modified Eagle medium (DMEM, high glucose, no glutamine; Gibco, Carlsbad, CA) supplemented with 10% tetracycline tested fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA), 2 mM L-glutamine (Gibco, Carlsbad, CA), and 100 U/mL penicillin-streptomycin (Gibco, Carlsbad, CA). The cells were incubated at 37 °C, 5% CO_2, and 98% humidity.

Transfection and receptor activation assays. HEK293 cells were seeded in white tissue culture treated 96-well plates with a clear flat bottom (Corning, NY, USA) at a density of 6,000 cells/well. After incubation overnight and at >70% confluence, cells were transiently transfected with lipofectamine (Invitrogen, Carlsbad, CA) and three cDNAs encoding for (i) receptor of interest (GLP-1R, GIPR, GLP2, or GCGR), (ii) CRE_{6x} promoted firefly luciferase reporter gene, and (iii) constitutively active CMV promoted β-galactosidase control. The cDNAs were combined in tube A and diluted with reduced serum Opti-MEM (Gibco, Carlsbad, CA) while lipofectamine was diluted in tube B with Opti-MEM. After careful mixing, tube A was transferred to tube B, vortexed, and incubated at room temperature for 45 minutes (method A) or 5 minutes (method B). With gentle pipetting, the transfection mixture was diluted a factor of 10 with serum free DMEM supplemented with L-glutamine and penicillin-streptomycin. The growth medium was aspirated from the microtiter plate and 50 µL of the reduced serum transfection mixture was distributed in each well with a final concentration of 0.2 µL/well lipofectamine, 10 ng/well receptor cDNA, 5 ng/well CRE_{6x}-luciferase cDNA, and 5 ng/well (method A) or 50 ng/well (method B) CMV-β-galactosidase.

Peptides of interest were serially diluted in serum free medium 18-24 hours post transfection. Each dilute peptide solution (or serum-free medium control) was pipetted into the corresponding well (50 µL/well) to final concentrations ranging from 1 µM to 1 pM. After 4-6 h incubation at 37 °C, 5% CO_2, and 98% humidity, medium was carefully aspirated from the cells and luciferase activity was measured by luminescence via PerkinElmer Victor X3 plate reader after addition of SteadyLite reagent (PerkinElmer Life and Analytical Sciences, Waltham, MA). Following chemiluminescence, β-galactosidase activity in each well was quantified by addition of substrate o-nitrophenyl-β-galactoside (50 µL, 13 mM, Thermo Scientific Pierce, Waltham, MA) in buffer and β-mercaptoethanol (80 µL, 51 mM, Aldrich, St. Louis, MO) in buffer. The production of *o*-nitrophenol was quantified by measuring the change in absorbance at 420 nm before and after 2-hour incubation at 37°C. The activity of β-galactosidase was used to correct the corresponding luciferase luminescence for differences in transfection efficiency and/or cell viability. All corrected luminescence was normalized to controls and fit to a sigmoidal dosed-response curve in GraphPad Prism v8 to calculate the EC_{50} values of each tested peptide.

***In vitro* degradation assays.** The stability of native and modified peptides was determined in presence of DPP4, FAP, or DPP9 (when applicable). For all reactions, peptides (0.1 mM) were incubated at 37 °C with and without proteases: hDPP4 (R&D Systems), DPP9 (R&D Systems), or FAP (R&D Systems) in reaction buffer (20 mM TRIS, 100 mM NaCl, 1 mM EDTA, pH 8.0). After 18 hours, the reactions were centrifuged and analyzed directly by LC-MS/MS or serially diluted with serum-free DMEM. The diluted reaction mixtures were added directly to transfected cells and the production of luciferase and β-galactosidase were quantified as outlined in receptor activation assays.

Steady State Kinetics with DPP4. All reagents were pre-warmed to 37 °C. Inhibitors or peptides were pre-incubated with DPP4 (200 mU, Pro-Spec) for 30 minutes before the addition of the chromogenic substrate, Gly-Pro-pNA (Carbosynth). The total concentration of DMSO in each reaction did not exceed 1%. The assay was initiated by addition of substrate in reaction buffer (20 mM TRIS, 100 mM NaCl, 1 mM EDTA, pH 8.0) at final reaction concentrations of 30 µM to 1000 µM. The progress of the DPP4 catalyzed reaction was monitored by production of *p*-nitroanaline by absorbance at 405 nm at 3 second intervals over 200 minutes with a Varioskan LUX multimode microplate reader (Thermo Fisher Scientific, Waltham, MA). Prism 8 was used to fit the initial velocity of each reaction, spanning no longer than 8 minutes. These Michaelis-Menten kinetics experiments were

used to calculate initial velocity (V_0) of the reactions, and was the plotted vs. initial substrate concentration ($[S]_0$) to compute K_m and V_{max} according to the following equation: $V_0 = V_{max}[S]_0/(K_m+[S]_0)$.

***In vivo* glucose tolerance test.** Thirteen-week-old male C57BL/6J mice (Charles River Laboratories) were fasted overnight prior to experiment. Blood glucose levels were monitored from a tail bleed using a blood glucose meter. The fasting blood glucose level was measured 75 minutes prior to first glucose challenge. Peptides were dissolved in sterile DMSO (Sigma) at a concentration of 10 mg/mL and further diluted 20-fold with TBS buffer (pH 7.4) to a final DMSO concentration of less than 5%. One hour prior to glucose injection, peptides were administered by intraperitoneal (i.p.) injection to mice at a 1 mpk dose using an injection volume of 10 mL/kg. Control mice were injected with vehicle (TBS) instead of peptide. At t= 0 min glucose levels were measured and a sterile 30% (w/v) D-glucose/saline solution was administered by i.p. injection at a 1.5 g/kg dose and 5 mL/kg volume. Blood glucose levels were measured at 30, 60, 90, and 120 minutes post glucose injection. Five hours after peptide injection, the mice were injected a second time with 1.5 g/kg glucose and blood glucose was monitored 30 minutes post second injection. After the glucose tolerance test was concluded, mice were sacrificed by CO_2 inhalation.

Serum stability assay. A central vein catheter (Tygon microbore tubing, Cole Parmer) was surgically inserted into male Sprague-Dawley rats (Charles River Laboratories) (n = 3) 2–3 cm into right vein jugularis under anesthesia (isofluorane) and pre- and post-surgery s.c. analgesic administration (Anorfin 0.05 mg/kg; Rimadyl Vet 5 mg/kg). The animals were allowed to recover for one-week post-surgery prior to experiment. On experiment day one, an i.v. bolus of 15 μL of 5 nmol/kg test compound (1 mL/kg) was given via the catheter followed by 0.2 mL of sterile saline. Blood was collected from vena sublingualis at various time points in ice cooled EDTA tubes, centrifuged for 5 min (4500 **g*) at 4 °C. Plasma was transferred and immediately frozen for later analysis by receptor stimulation bioassay.

Statistical analysis. Error bars and values of *n* are reported in figure captions and tables. Prism 8.0 (GraphPad) was used to generate and fit concentration-response curves and perform an ordinary ANOVA followed by Tukey post hoc test. *In vitro* experiments were blinded, and sample sizes were not predetermined by statistical analysis.

References

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(8) Fortin, J. P.; Schroeder, J. C.; Zhu, Y. T. E.; Beinborn, M.; Kopin, A. S. Pharmacological Characterization of Human Incretin Receptor Missense Variants. *J. Pharmacol. Exp. Ther.* **2010**, *332*, 274-280.
(9) Fortin, J. P.; Zhu, Y. T.; Choi, C.; Beinborn, M.; Nitabach, M. N.; Kopin, A. S. Membrane-tethered ligands are effective probes for exploring class B1 G protein-coupled receptor function. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106*, 8049-8054.

Table S1: Analytical Characterization of Synthesized Peptides[a]

Peptide	MW (Calculated)[b]	MW (Found)[c]
GLP-1	3297.68	3298.05[d]
Liraglutide	3751.26	3751.07
1-GLP1	3338.72	3339.49
1-GIP	5025.64	5026.11
2-GLP1	3378.71	3378.85
2-Liraglutide	3833.26	3833.22
2-Exenatide	4267.70	4268.20
2-Glucagon	3563.83	3565.21
2-GIP	5064.63	5065.83
2-Triagonist(Ala2)	4612.05	4610.66
2-GLP2	3847.20	3847.16
2-Semaglutide(Ala2)	4180.61	4181.4
2-MetEnk	655.69	655.26
3-GLP1	3324.74	3325.21
4-GLP1	3338.77	3339.73
5-GLP1	3352.79	3353.26
6-GLP1	3478.73	3479.49
7-GLP1	3444.92	3446.99[d]
7-Glucagon	3630.06	3630.97
7-GLP2	3913.40	3927.49
8-GLP1	3462.90	3464.39
9-GLP1	3402.81	3403.83
10-GLP1	3386.81	3387.17
11-GLP1	3376.78	3377.98
12-GLP1	3353.78	3355.07
13-GLP1	3369.78	3370.68
14-GLP1	3369.78	3370.86
15-GLP1	3429.88	3430.86
16-GLP1	3429.88	3431.86
17-GLP1	3494.83	3495.29
18-GLP1	3378.79	3379.12
19-GLP1	3460.84	3455.70[d]

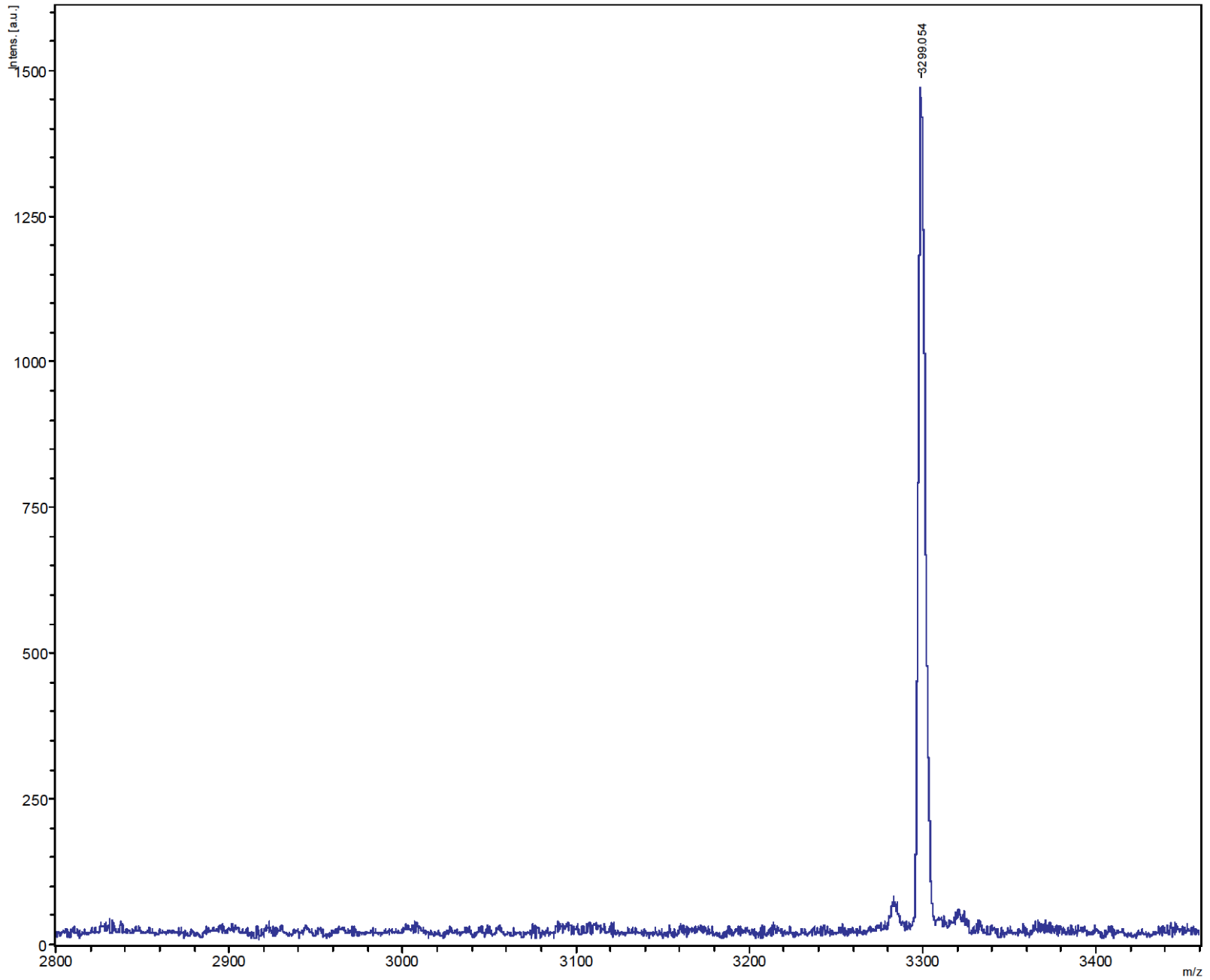
[a]All peptides were identified to be >95% pure by analytical RP-HPLC using a C18 column (Vydac, 5 μm, 4 mm x 250 mm) and solvents A (99% H_2O, 1% CH_3CN, 0.1% TFA) and B (10% H_2O, 90% CH_3CN, 0.07% TFA) with a linear gradient of 20-60% solvent B over 20 min at a flow rate of 1 mL/min and absorbance monitored at 230 nm.

[b]Molecular weight of each peptide was calculated from average masses using Peptide Mass Calculation v3.2 (Katholieke Univeriteit, Leuven, Belgium).

[c]Found molecular weights, unless specified otherwise, were detected by electrospray ionization-mass spectrometry (ESI-MS) of purified peptides dissolved in 50% H_2O/CH_3CN and 0.1% TFA and carried out a using direct infusion method.

[d]Molecular weight measured by MALDI-TOF MS of pure peptide dissolved in 50% H_2O/CH_3CN in α-cyano-4-hydroxycinnamic acid matrix, containing 0.1% TFA.

GLP1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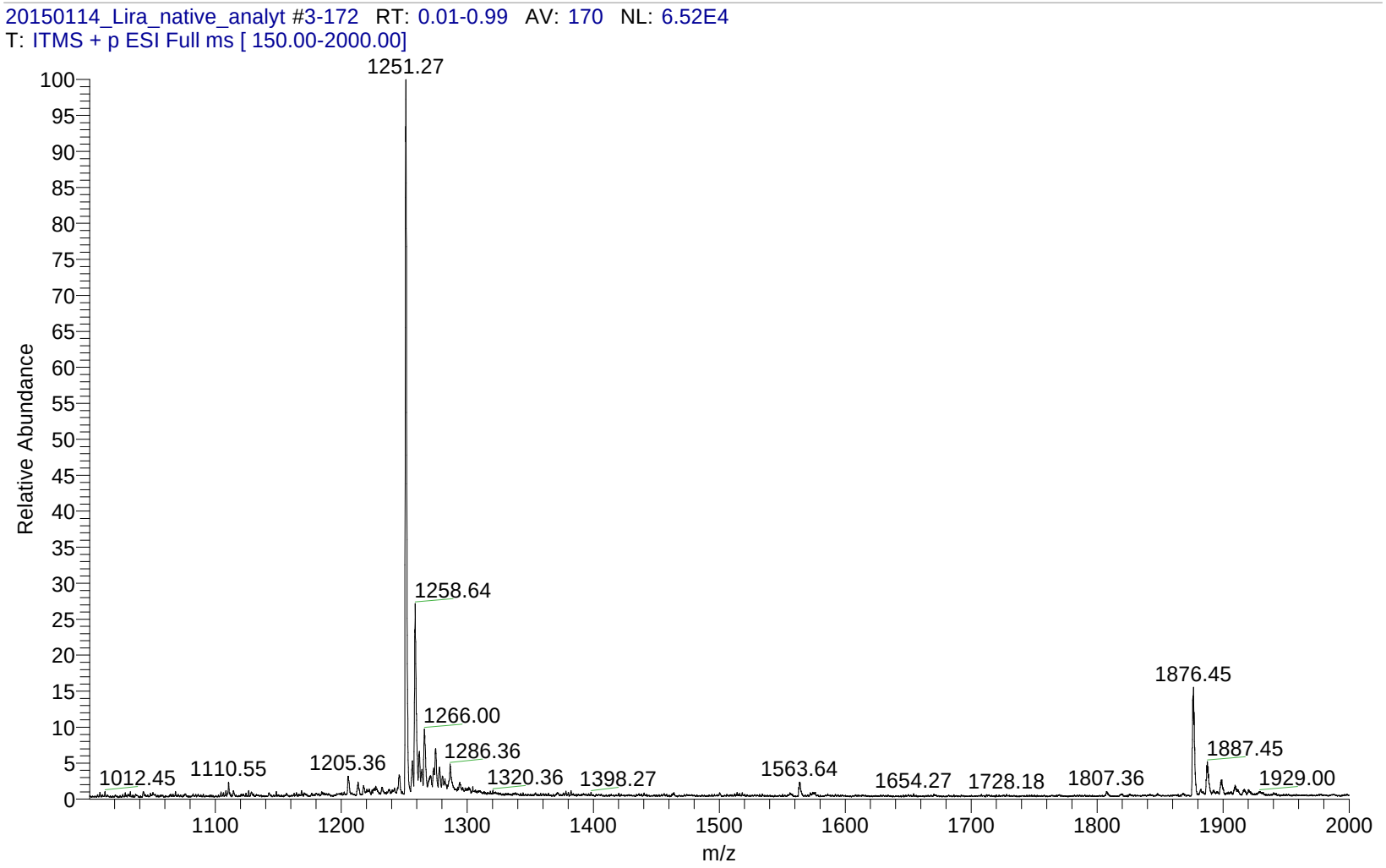
m/z

Liraglutide



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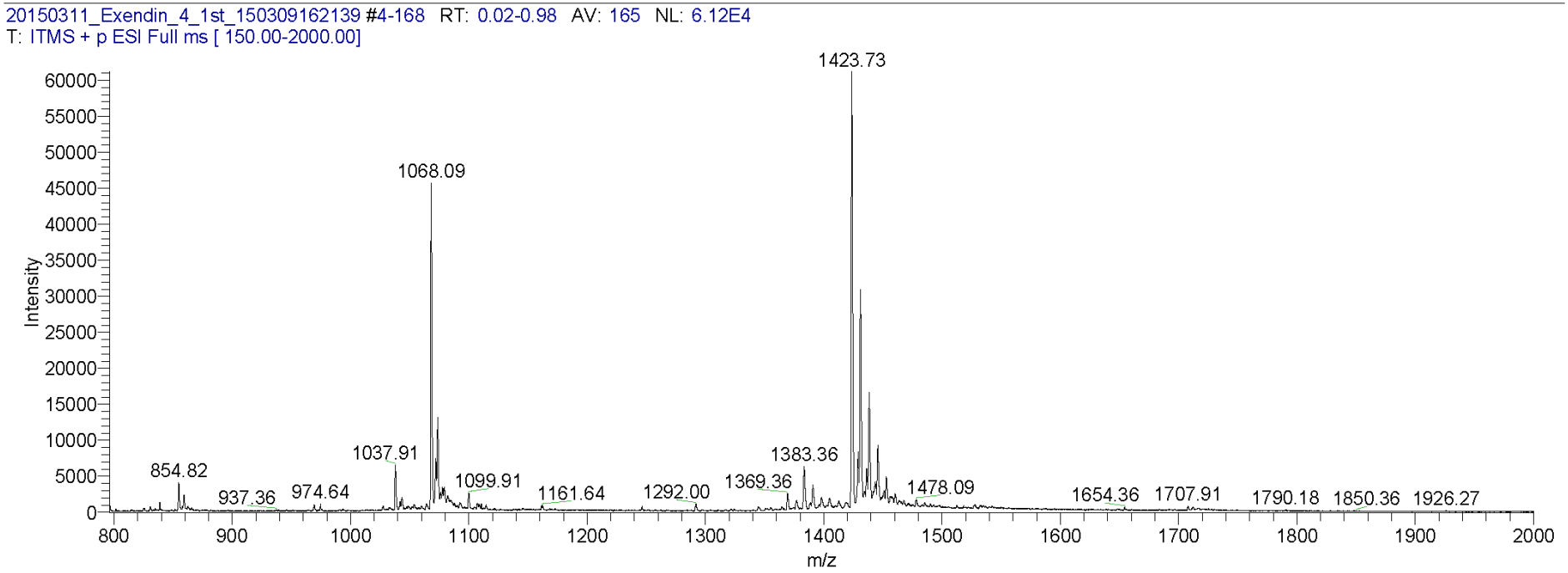
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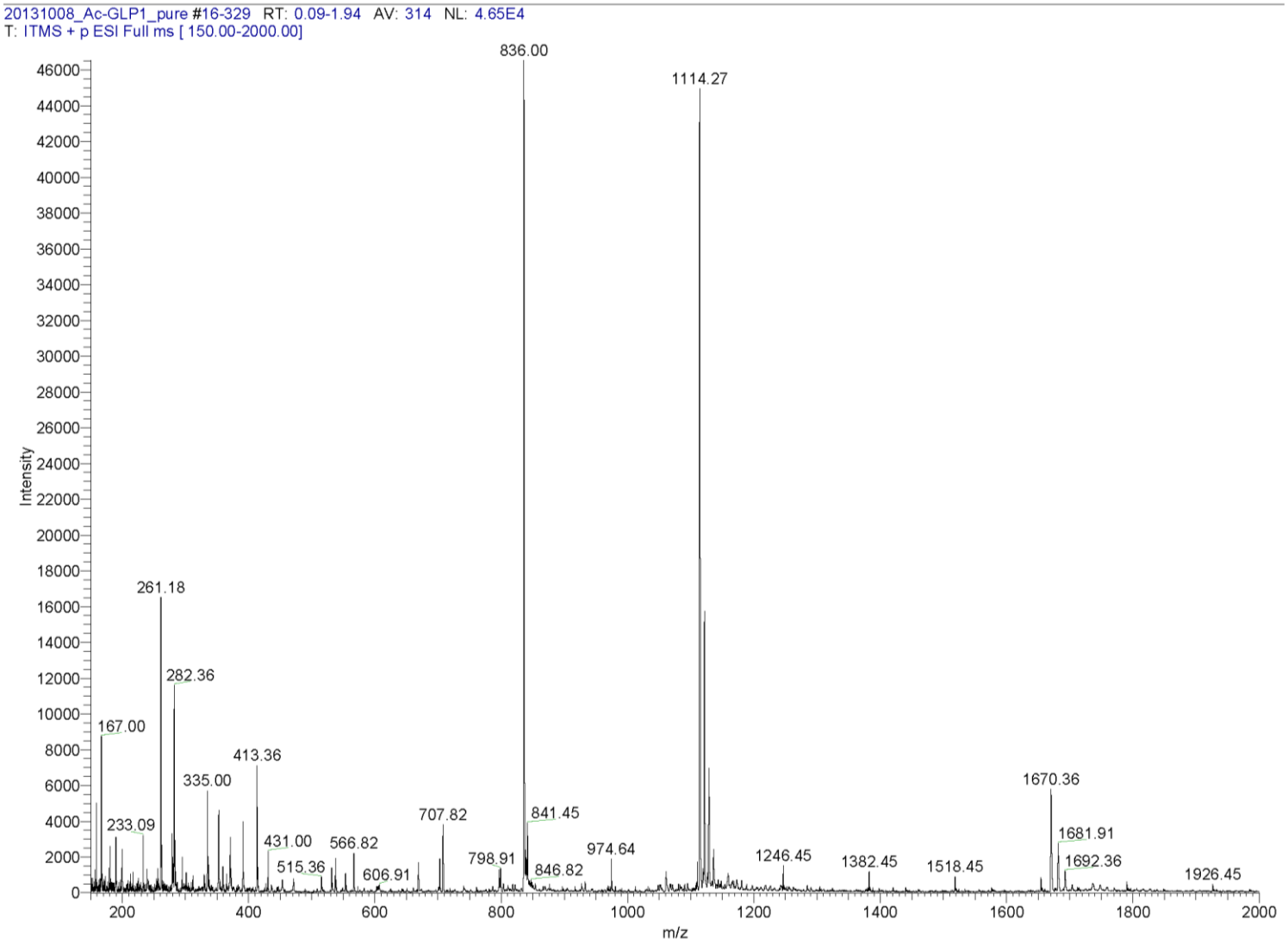
1926.27

1-GLP1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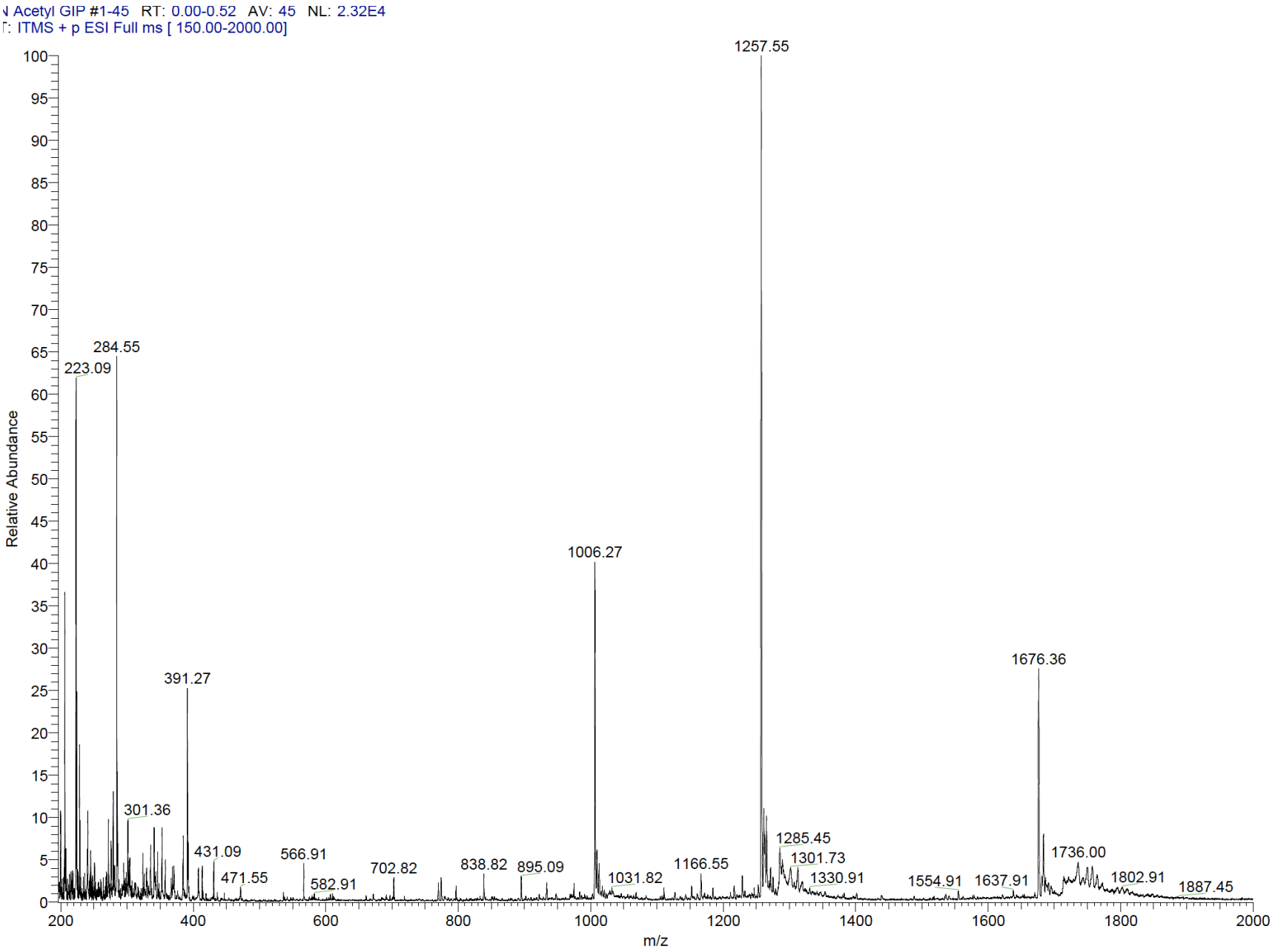
Intensity
m/z

1-GIP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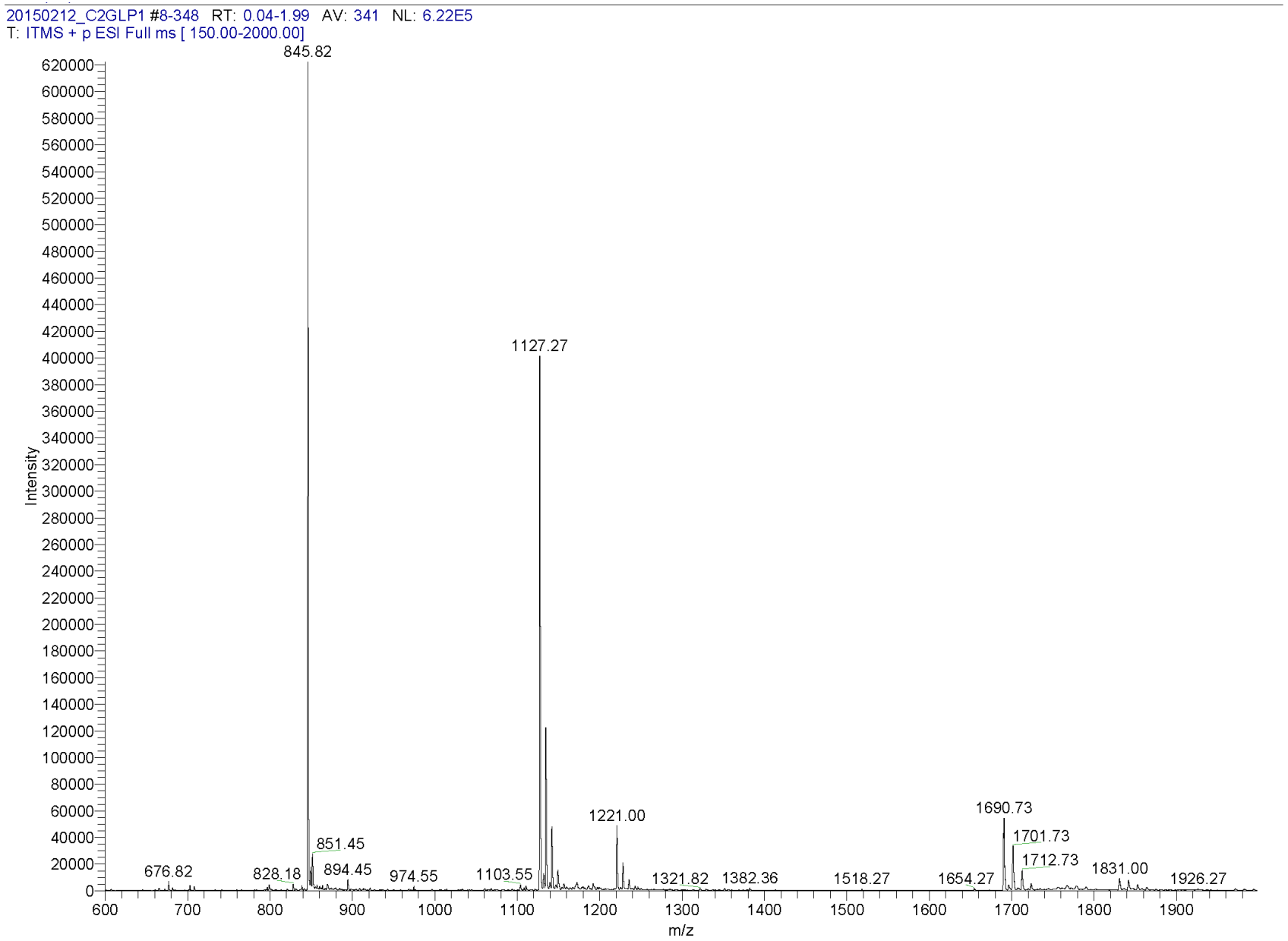
2-GLP1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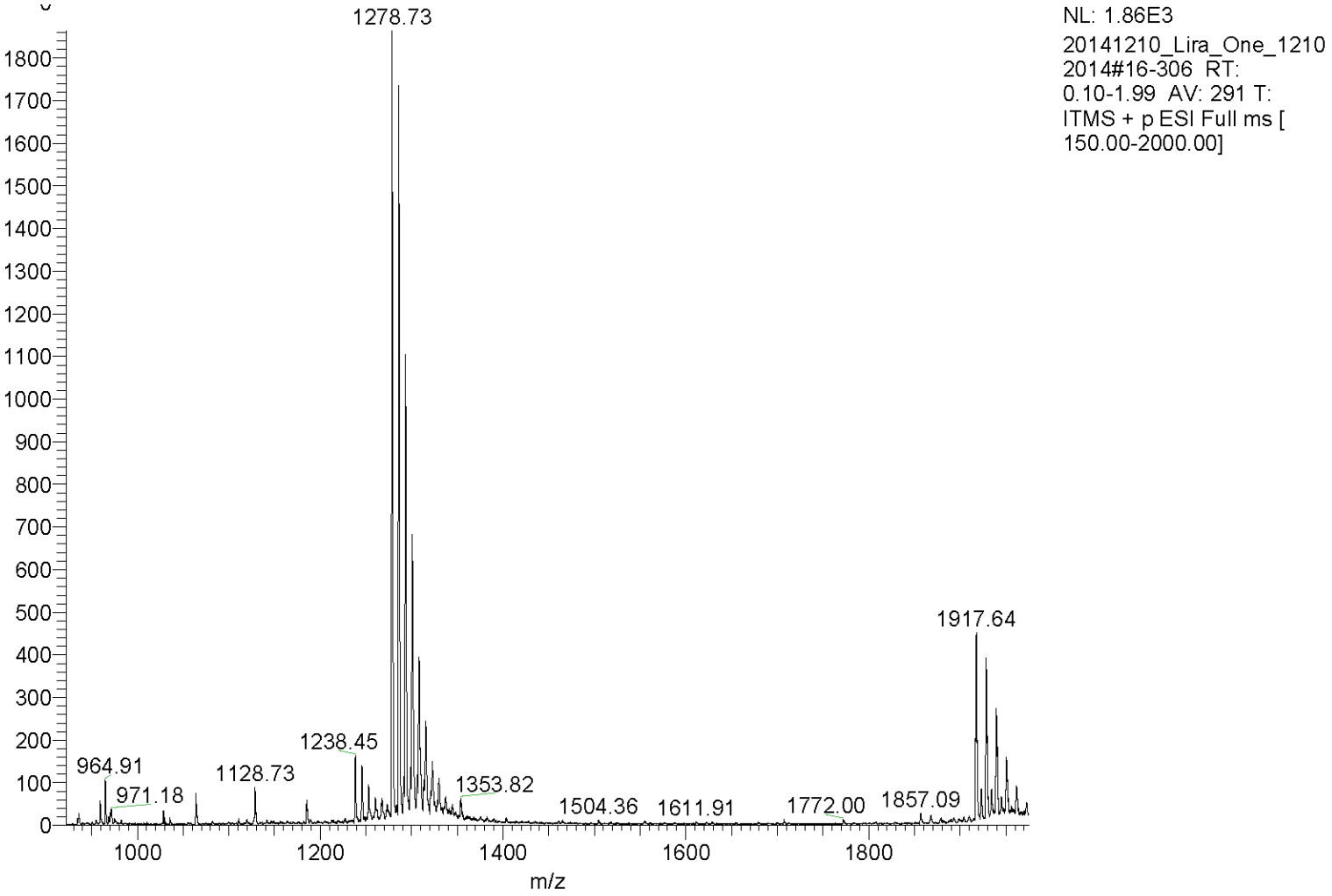

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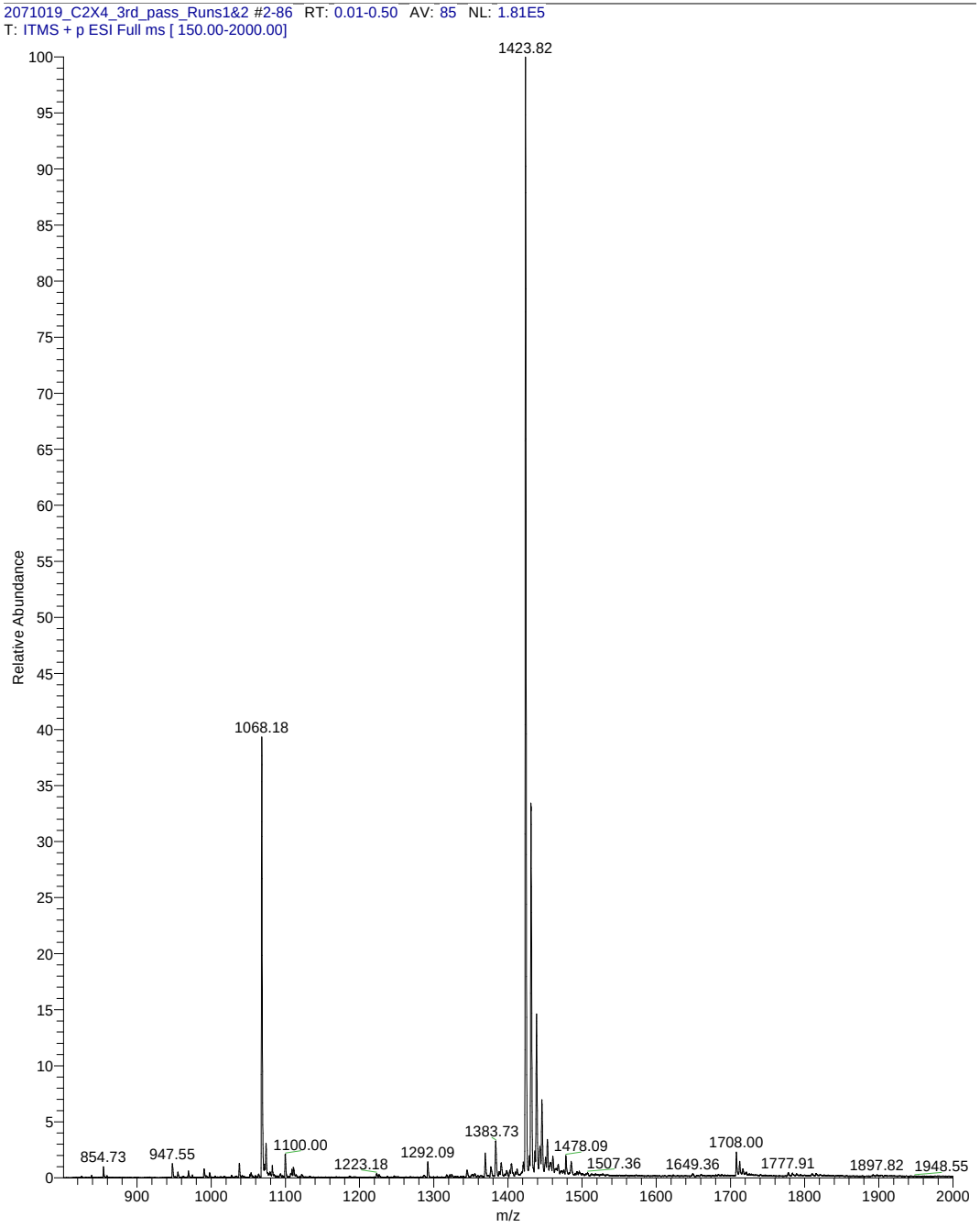
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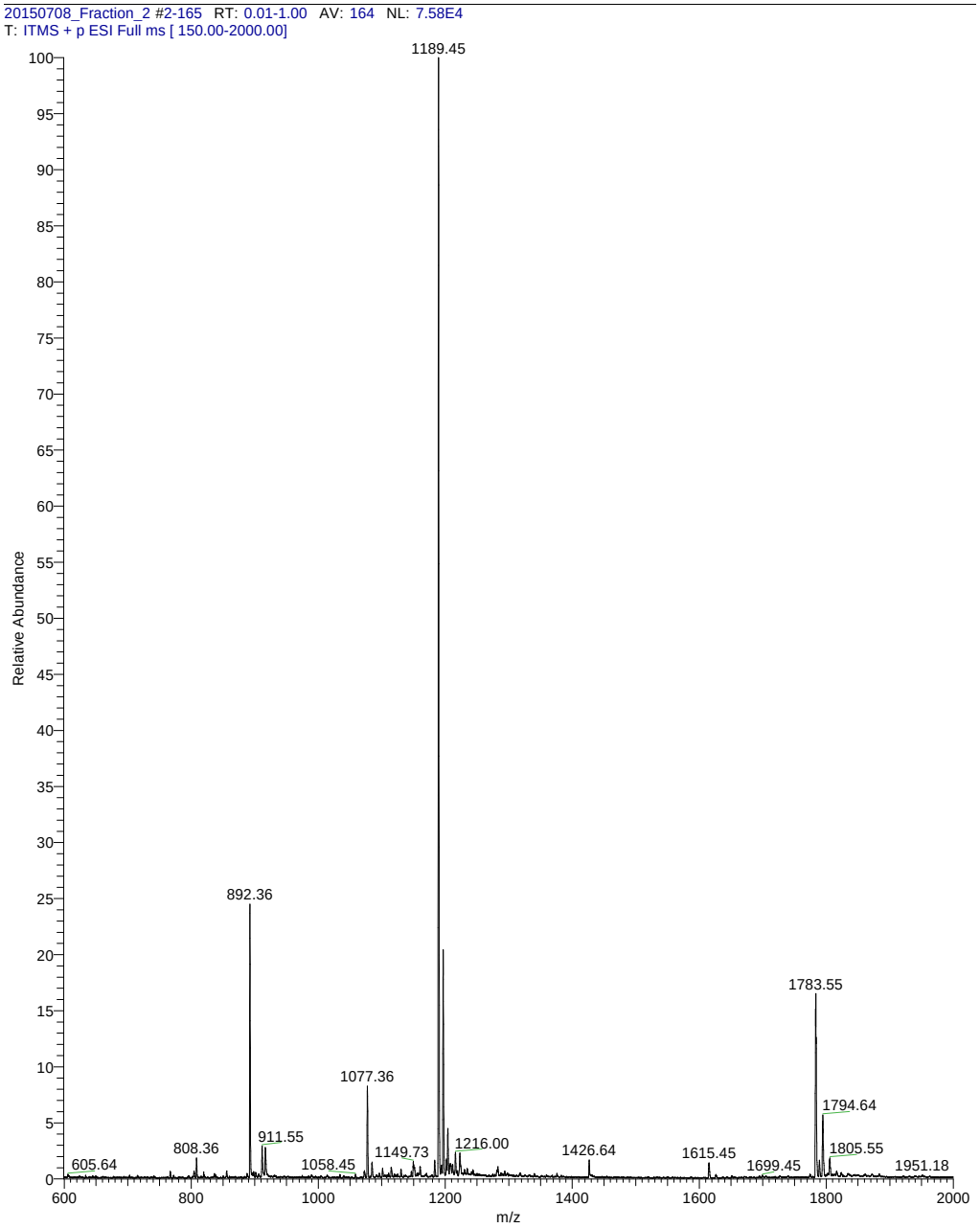
2-Glucagon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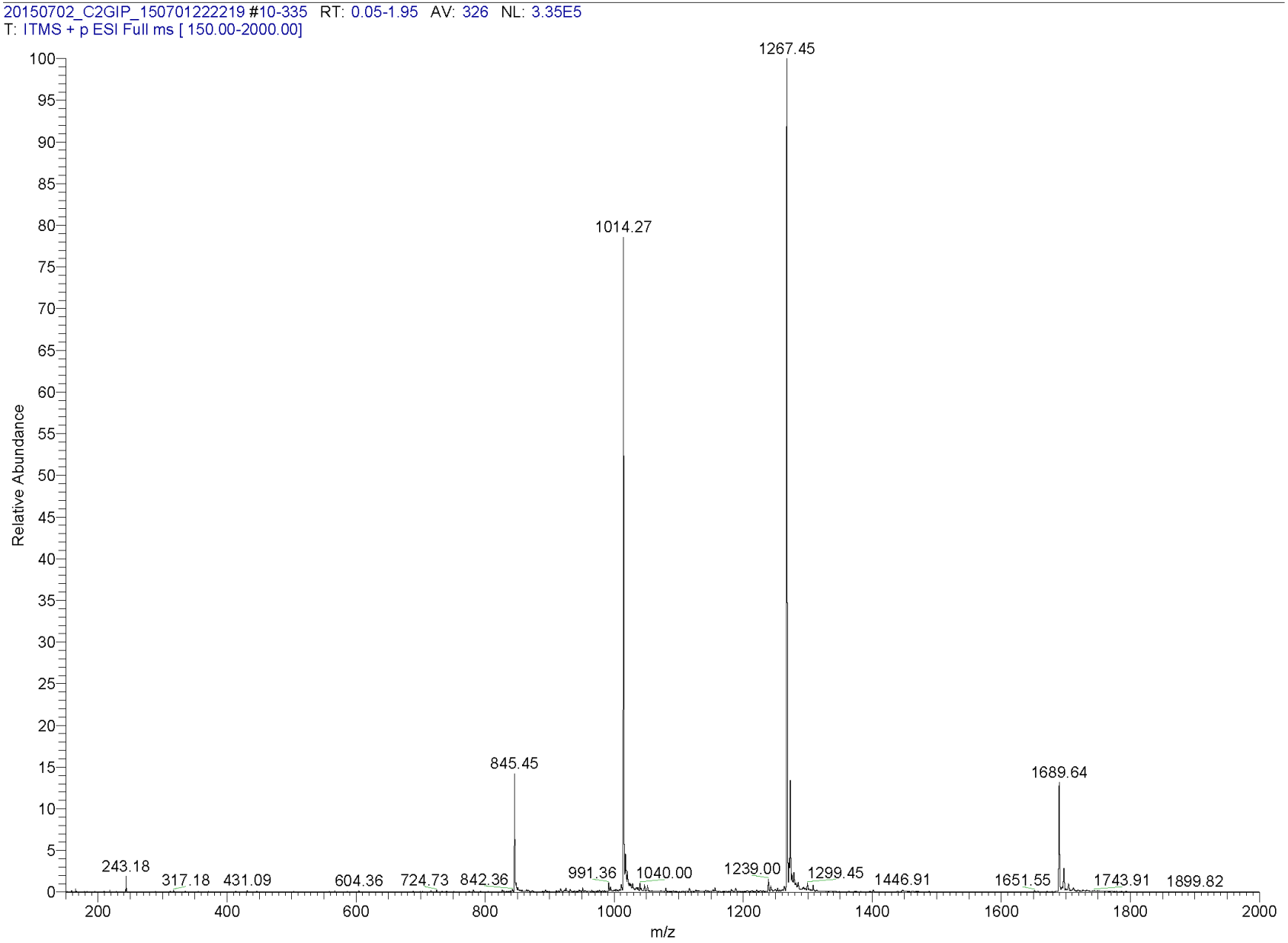
2-GIP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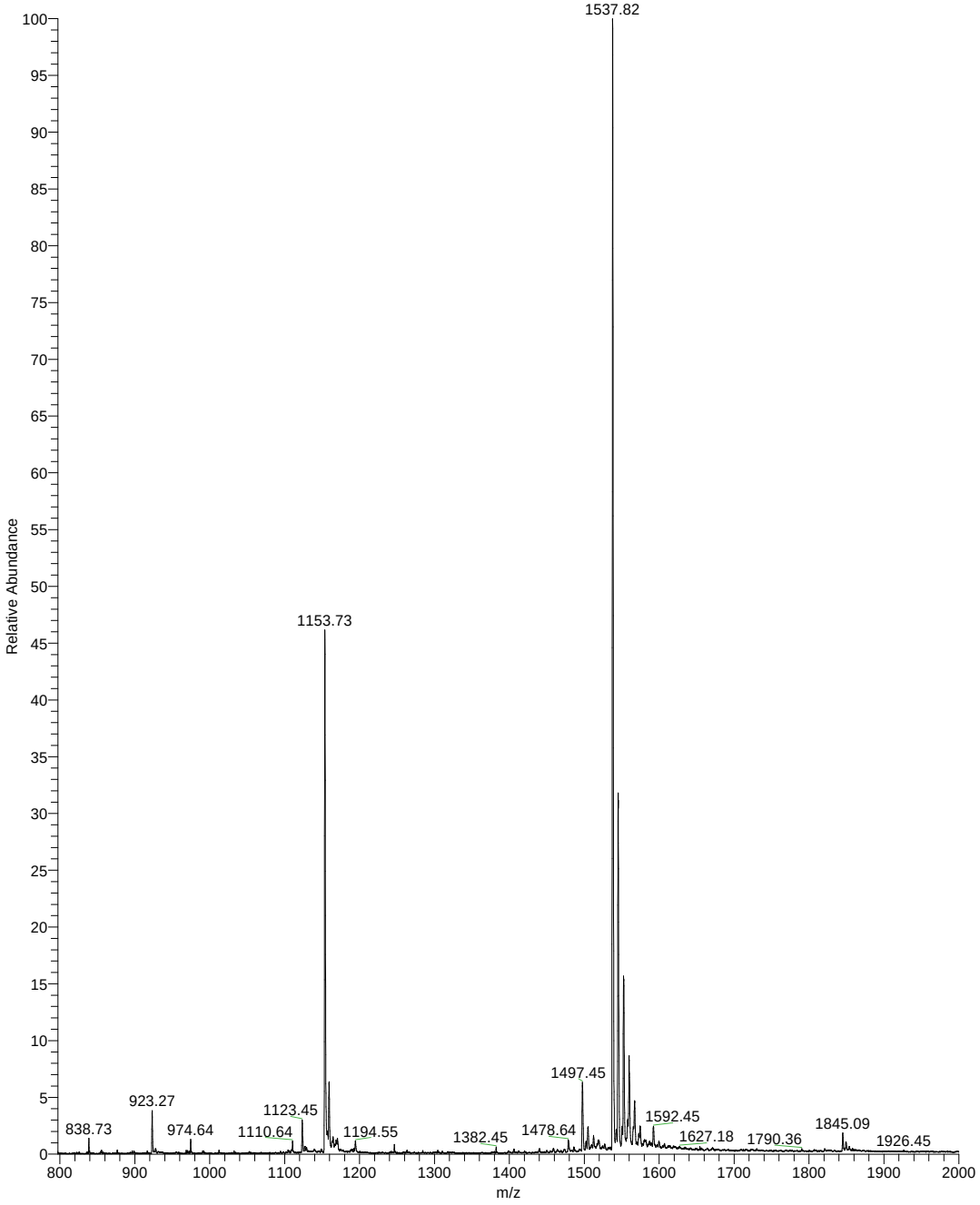
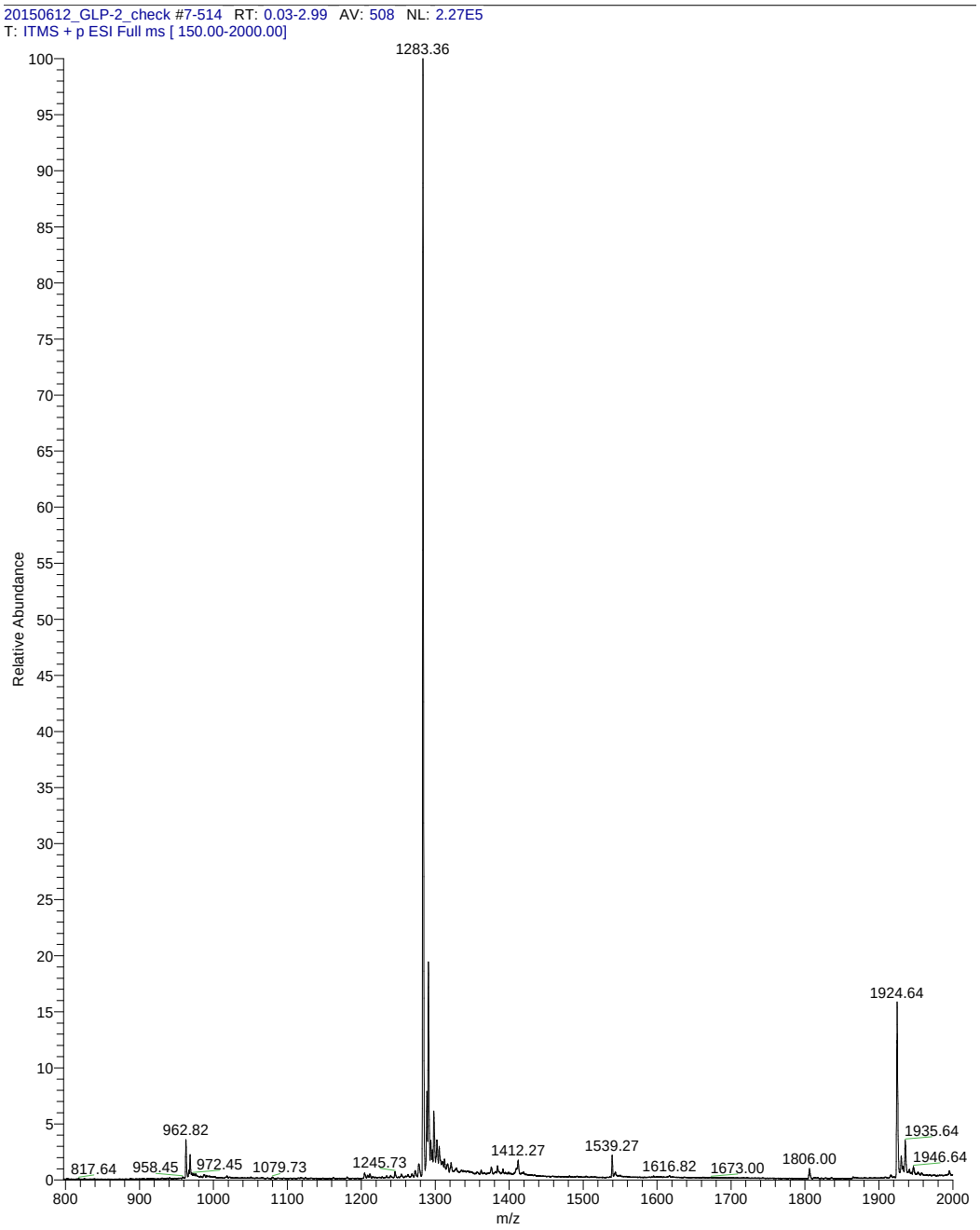
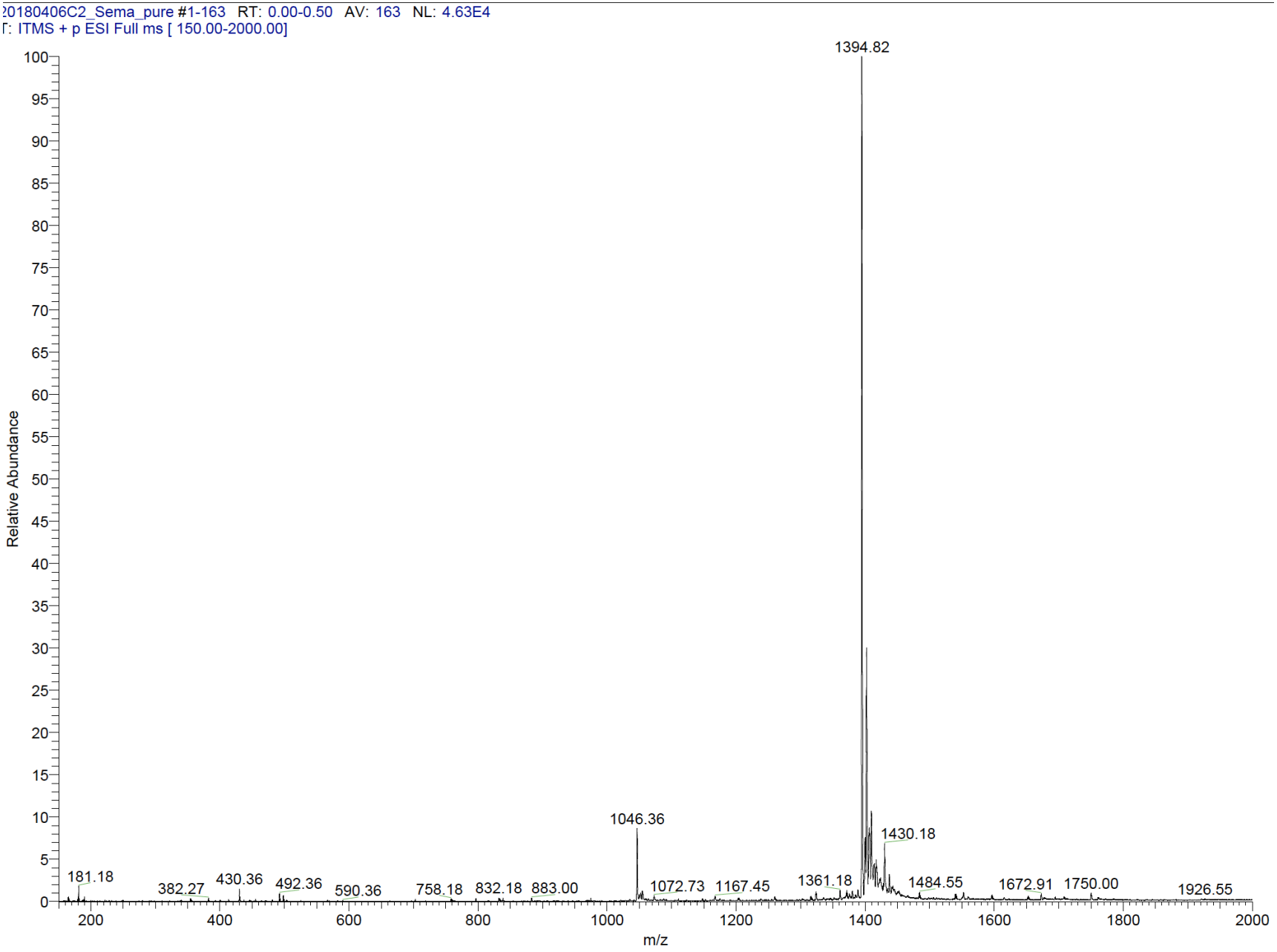


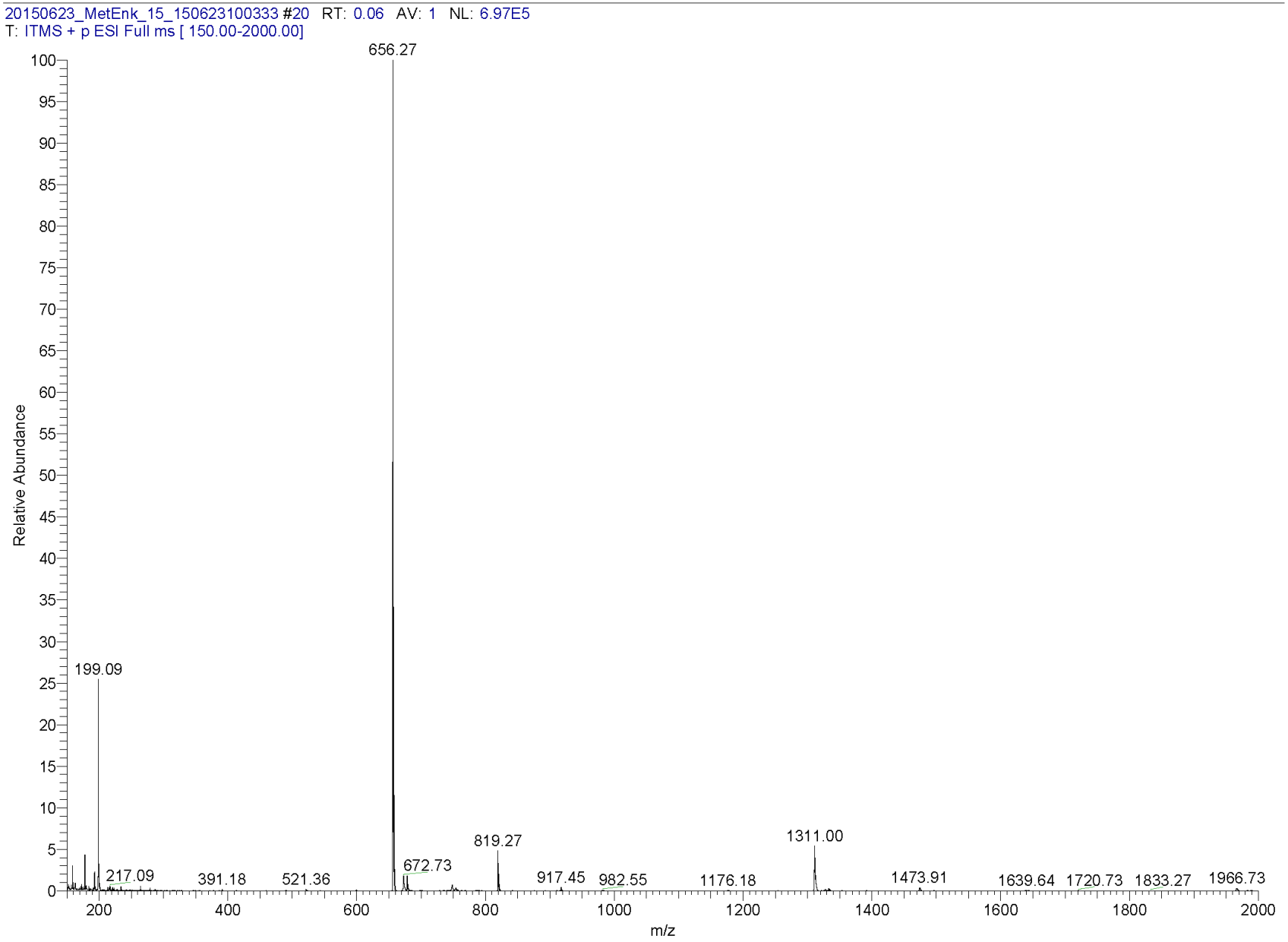
2-GLP2


20150612_GLP-2_check #7-514 RT: 0.03-2.99 AV: 508 NL: 2.27E5
T: ITMS + p ESI Full ms [150.00-2000.00]
Relative Abundance
m/z
1283.36
1924.64
962.82
1935.64
1946.64
1412.27
1539.27
817.64
958.45
972.45
1079.73
1245.73
1616.82
1673.00
1806.00

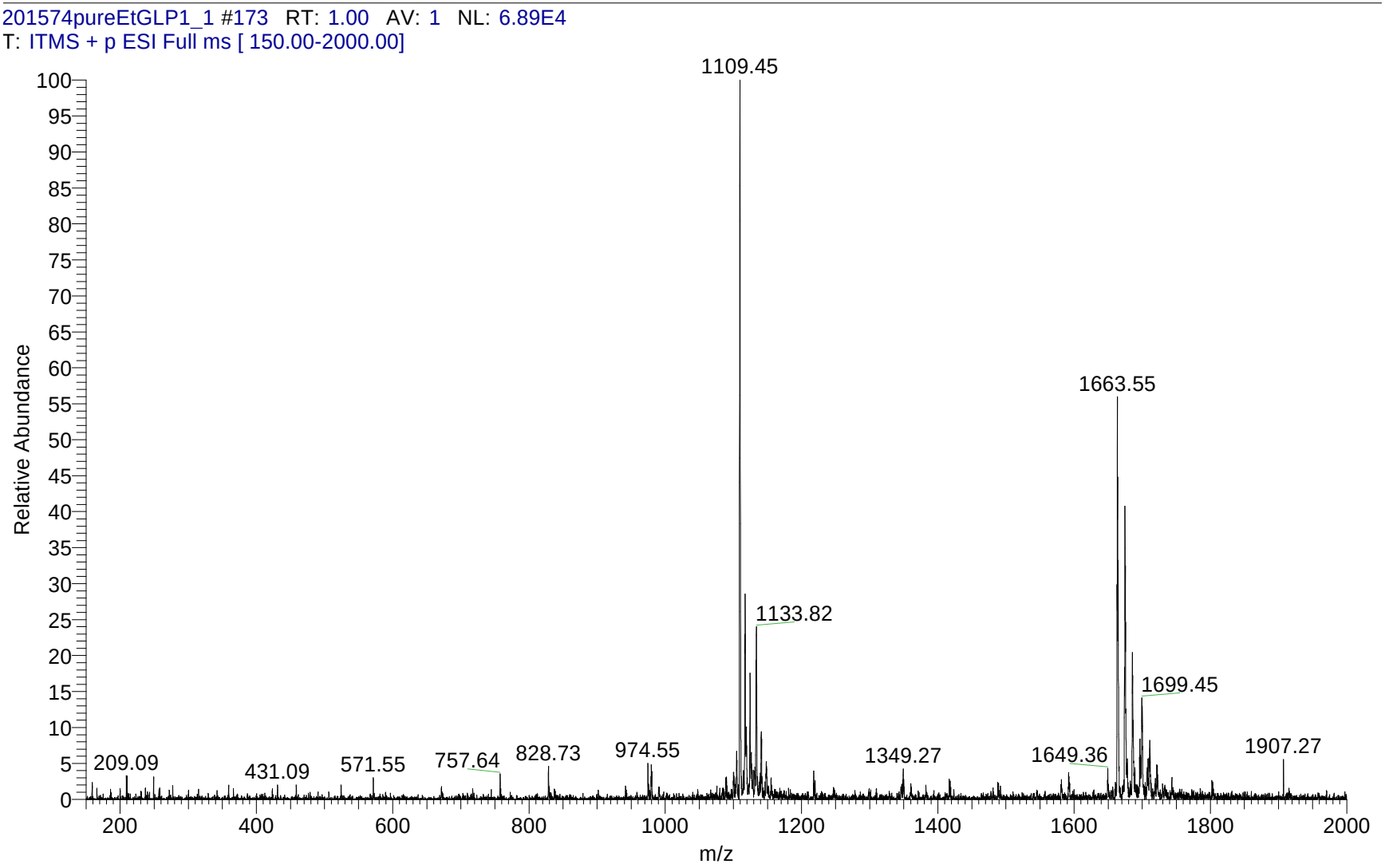
2-Semaglutide(Ala2)



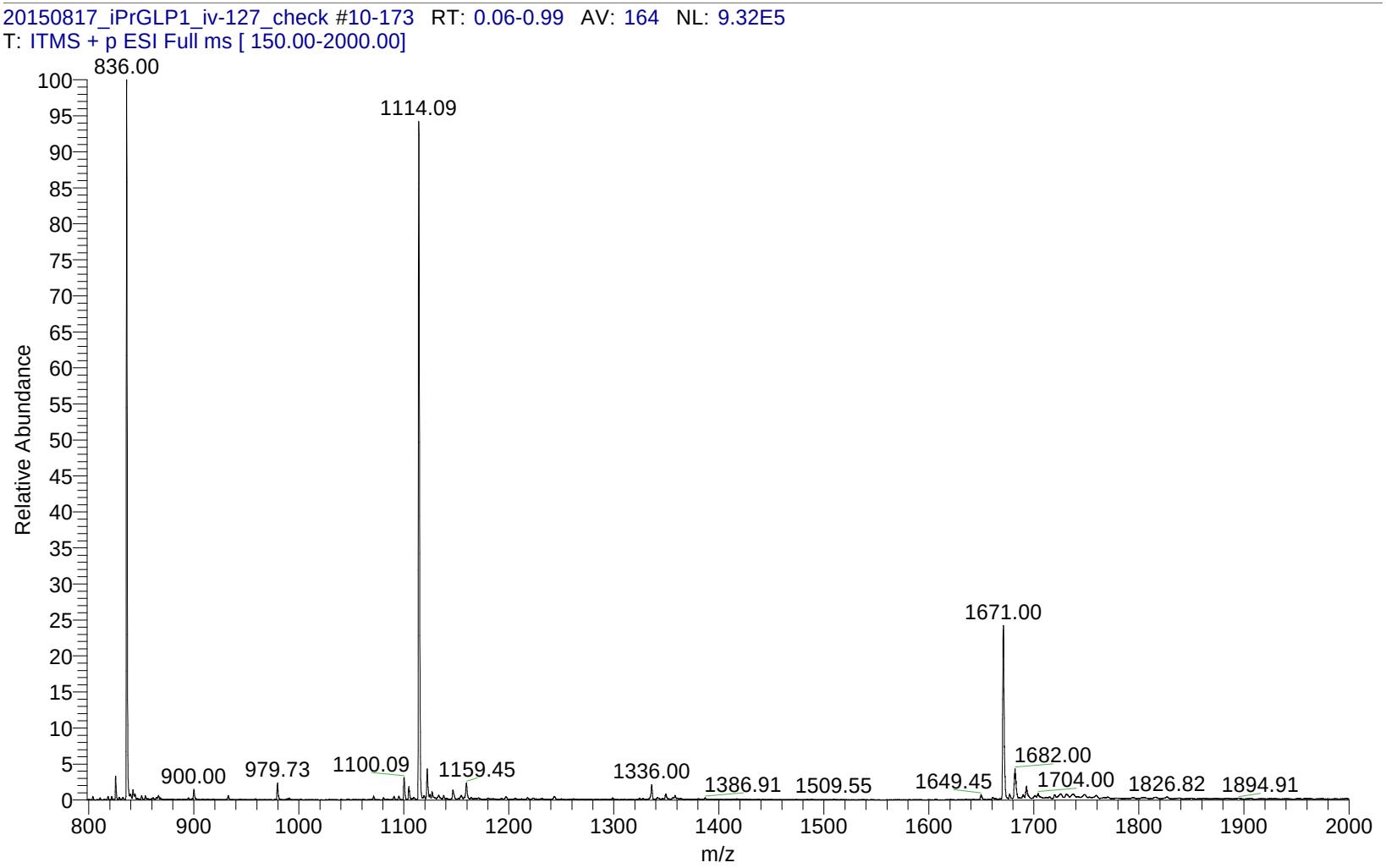
2-MetEnk


20150623_MetEnk_15_150623100333 #20 RT: 0.06 AV: 1 NL: 6.97E5
T: ITMS + p ESI Full ms [150.00-2000.00]
Relative Abundance
m/z
656.27
199.09
217.09
391.18
521.36
672.73
819.27
917.45
982.55
1176.18
1311.00
1473.91
1639.64
1720.73
1833.27
1966.73

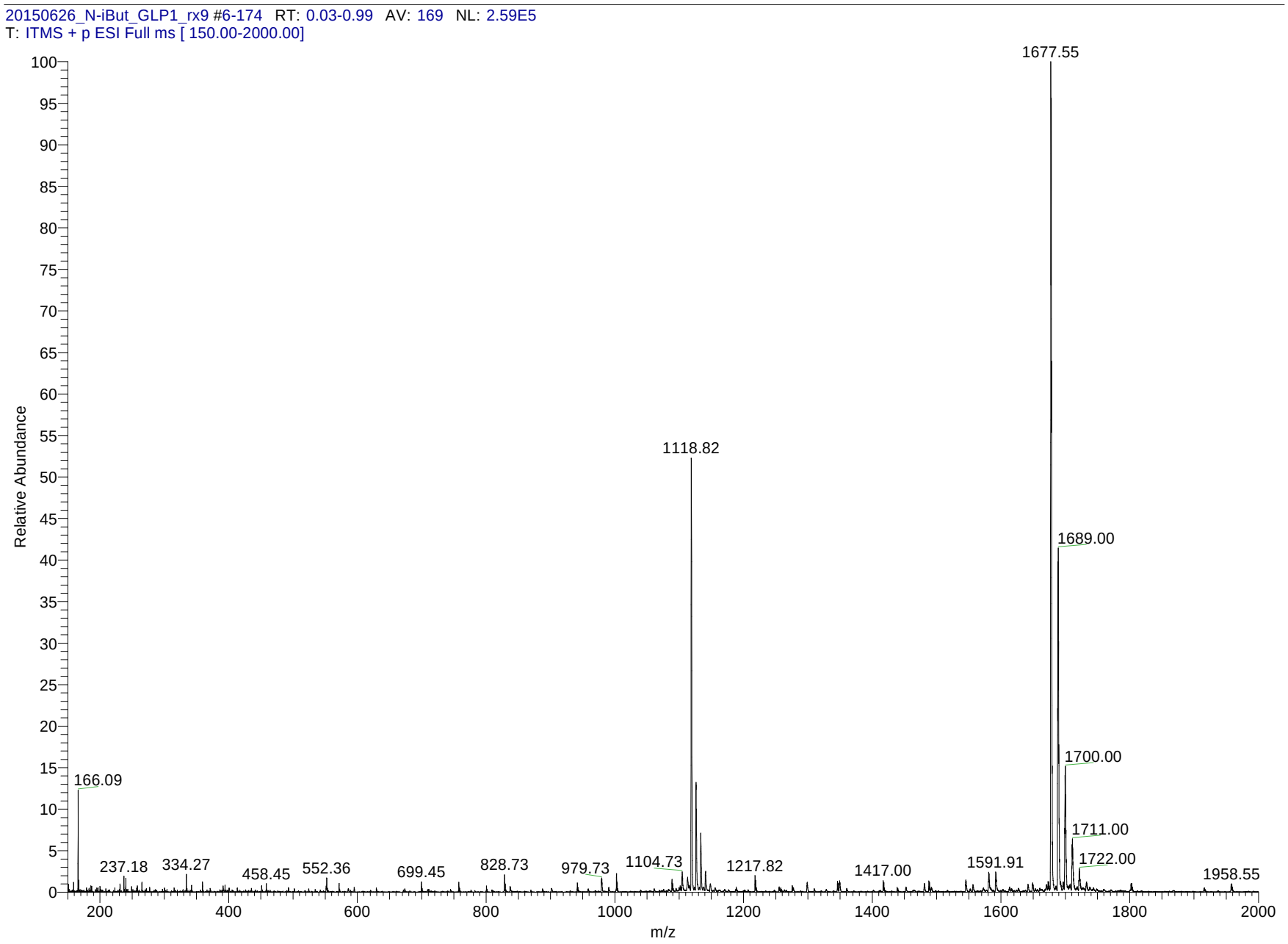
3-GLP1


201574pureEtGLP1_1 #173 RT: 1.00 AV: 1 NL: 6.89E4
T: ITMS + p ESI Full ms [150.00-2000.00]
Relative Abundance
m/z
1109.45
1663.55
1133.82
1699.45
209.09
431.09
571.55
757.64
828.73
974.55
1349.27
1649.36
1907.27

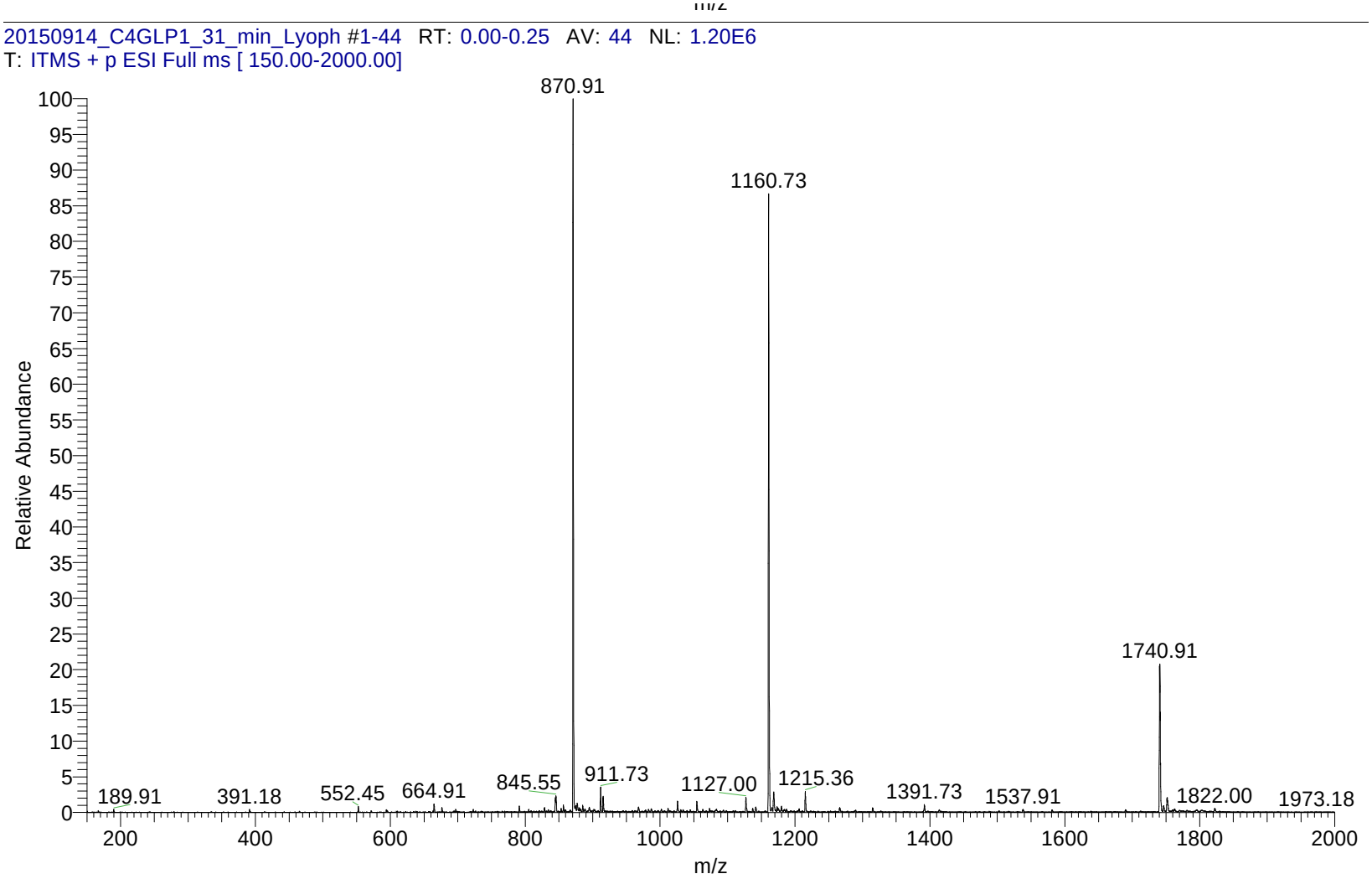
4-GLP1


20150817_iPrGLP1_iv-127_check #10-173 RT: 0.06-0.99 AV: 164 NL: 9.32E5
T: ITMS + p ESI Full ms [150.00-2000.00]
836.00
1114.09
1671.00
900.00
979.73
1100.09
1159.45
1336.00
1386.91
1509.55
1649.45
1682.00
1704.00
1826.82
1894.91
Relative Abundance
m/z

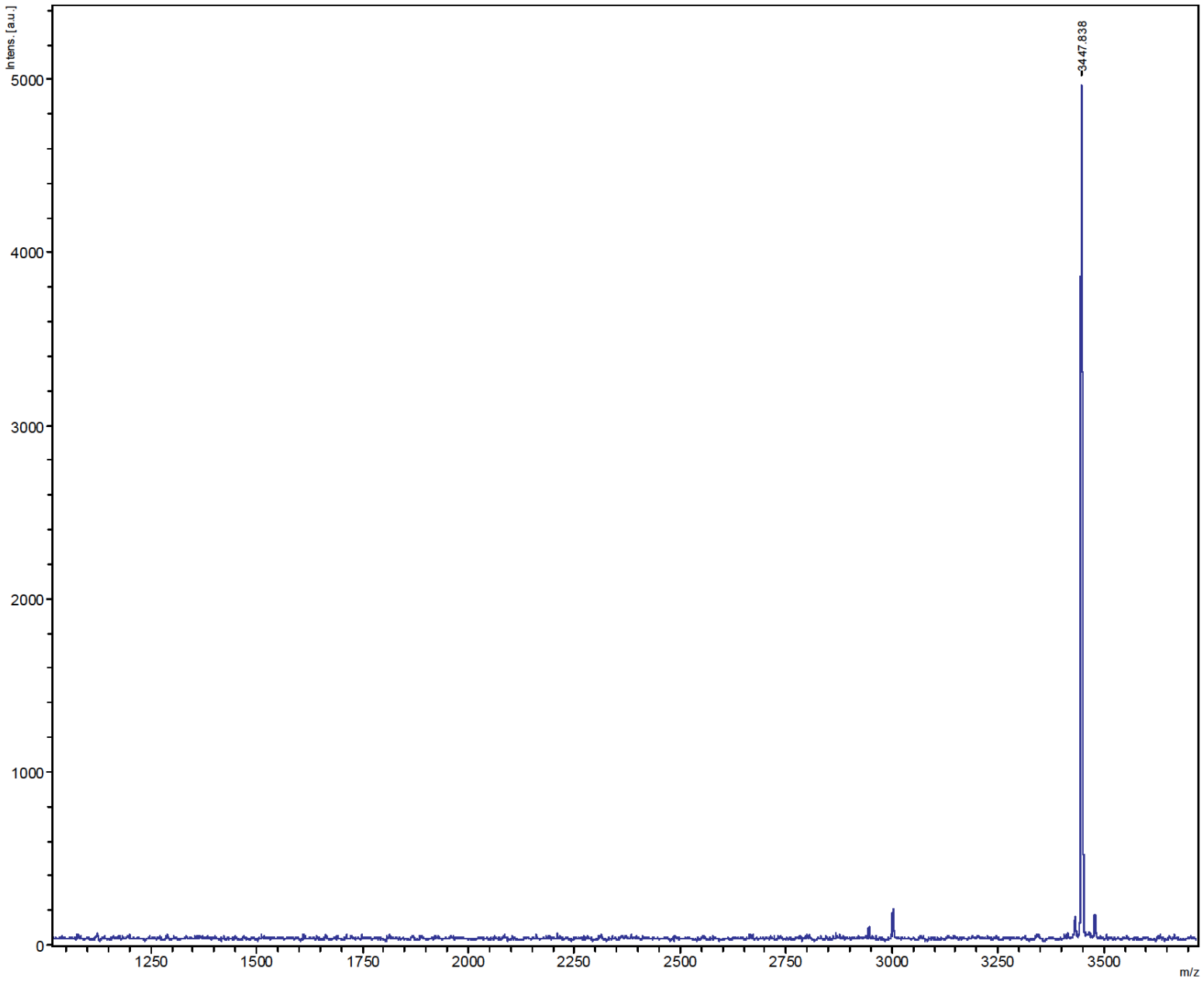
5-GLP1


20150626_N-iBut_GLP1_rx9 #6-174 RT: 0.03-0.99 AV: 169 NL: 2.59E5
T: ITMS + p ESI Full ms [150.00-2000.00]
Relative Abundance
m/z
1677.55
1118.82
1689.00
1700.00
1711.00
1722.00
166.09
237.18
334.27
458.45
552.36
699.45
828.73
979.73
1104.73
1217.82
1417.00
1591.91
1958.55

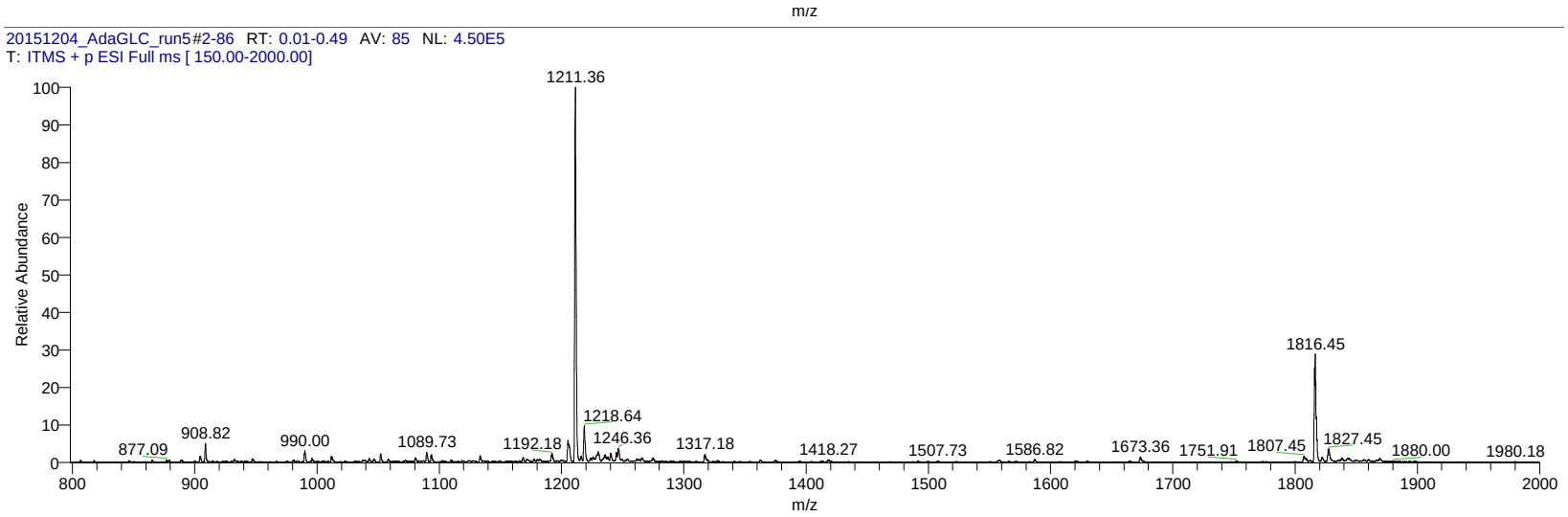
6-GLP1


m/z
20150914_C4GLP1_31_min_Lyoph #1-44 RT: 0.00-0.25 AV: 44 NL: 1.20E6
T: ITMS + p ESI Full ms [150.00-2000.00]
870.91
1160.73
1740.91
189.91
391.18
552.45
664.91
845.55
911.73
1127.00
1215.36
1391.73
1537.91
1822.00
1973.18
Relative Abundance
m/z

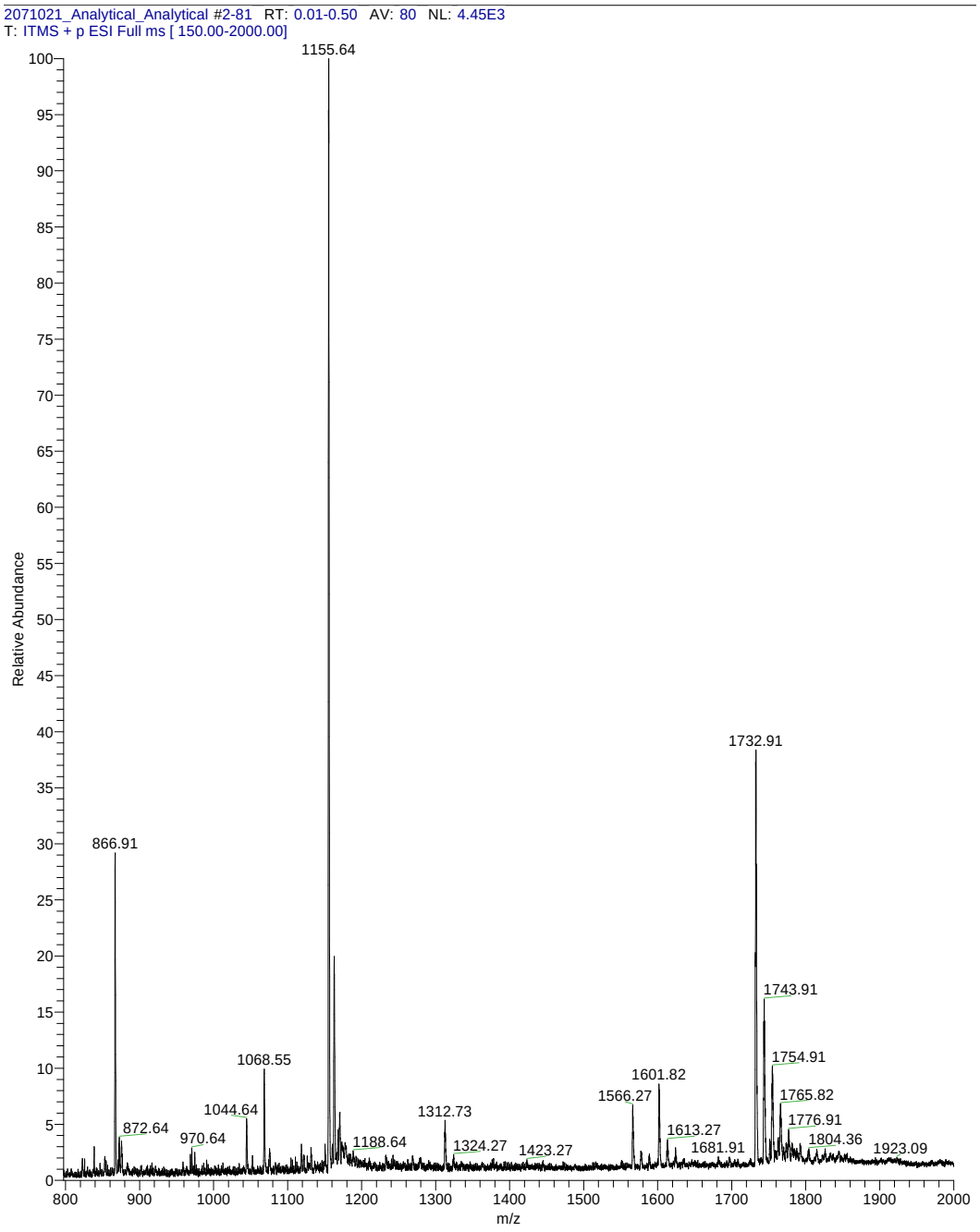
7-GLP1


Intens. [a.u.]
5000
4000
3000
2000
1000
0
3447.838
1250
1500
1750
2000
2250
2500
2750
3000
3250
3500
m/z

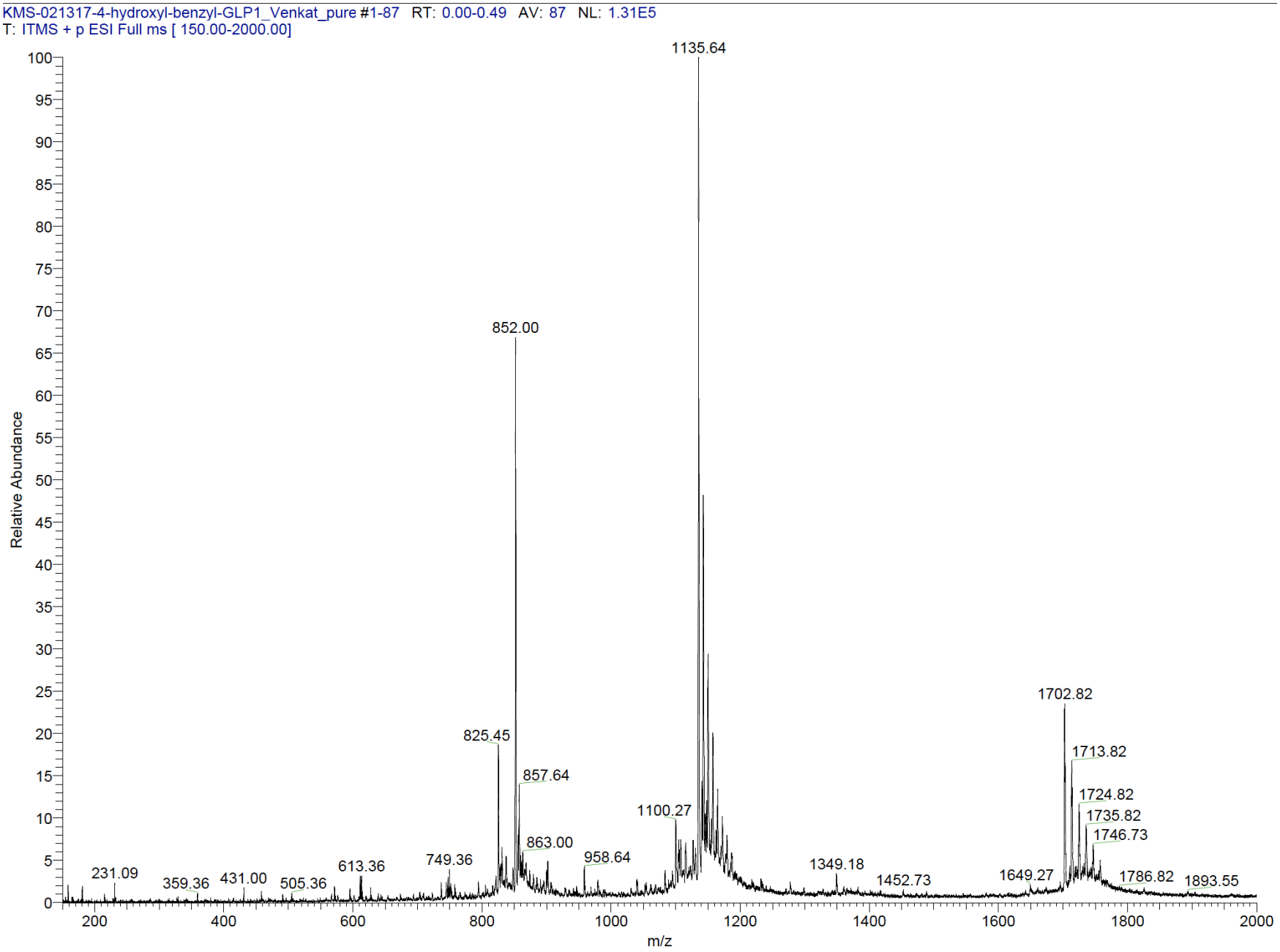
7-Glucagon


m/z
20151204_AdaGLC_run5#2-86 RT: 0.01-0.49 AV: 85 NL: 4.50E5
T: ITMS + p ESI Full ms [150.00-2000.00]
Relative Abundance
m/z
1211.36
1816.45
1218.64
1246.36
908.82
877.09
990.00
1089.73
1192.18
1317.18
1418.27
1507.73
1586.82
1673.36
1751.91
1807.45
1827.45
1880.00
1980.18

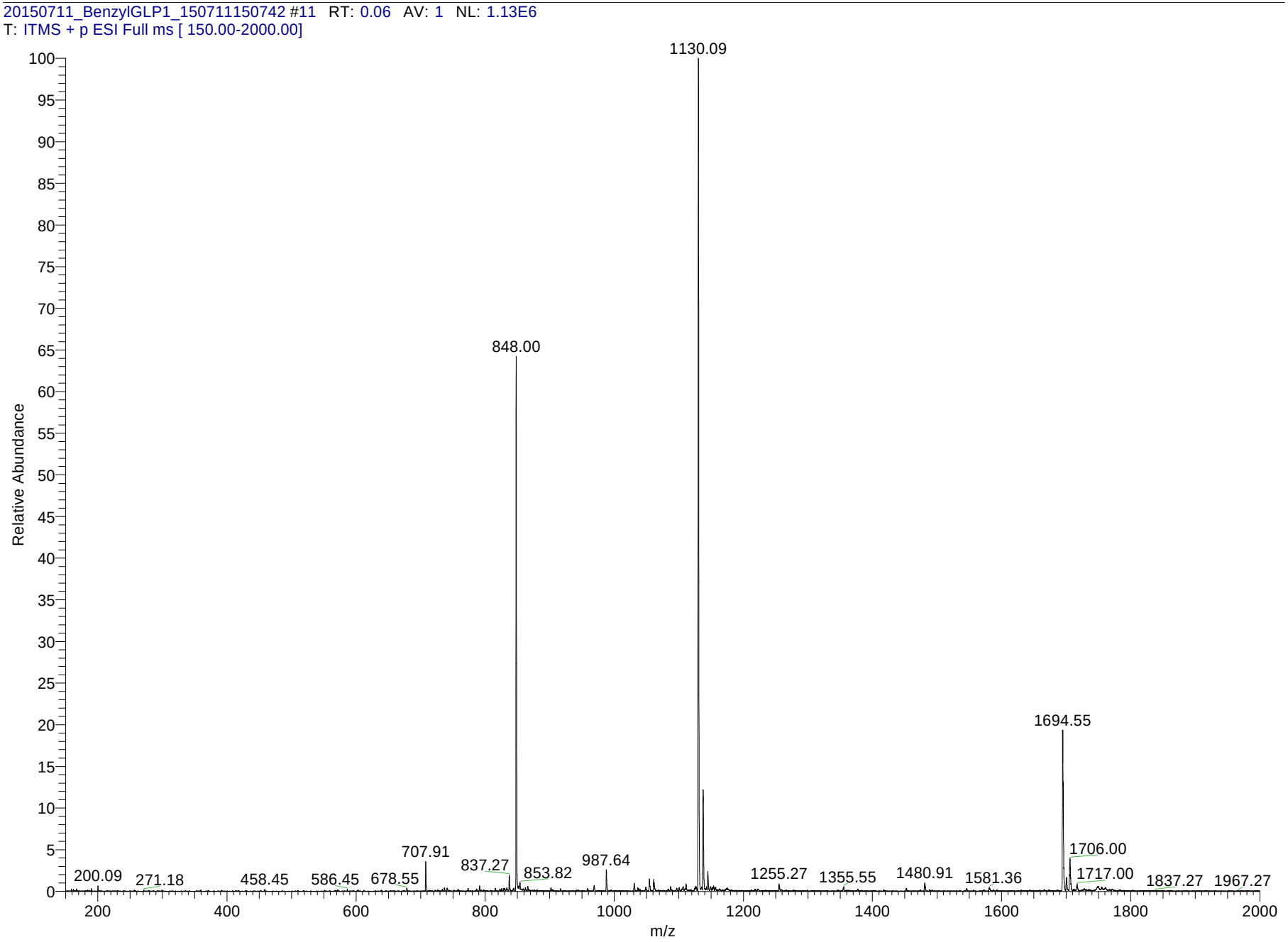
8-GLP1


2071021_Analytical_Analytical #2-81 RT: 0.01-0.50 AV: 80 NL: 4.45E3
T: ITMS + p ESI Full ms [150.00-2000.00]
Relative Abundance
m/z
1155.64
1732.91
866.91
1743.91
1754.91
1068.55
1601.82
1566.27
1765.82
1044.64
1312.73
1776.91
872.64
1613.27
970.64
1804.36
1188.64
1324.27
1423.27
1681.91
1923.09

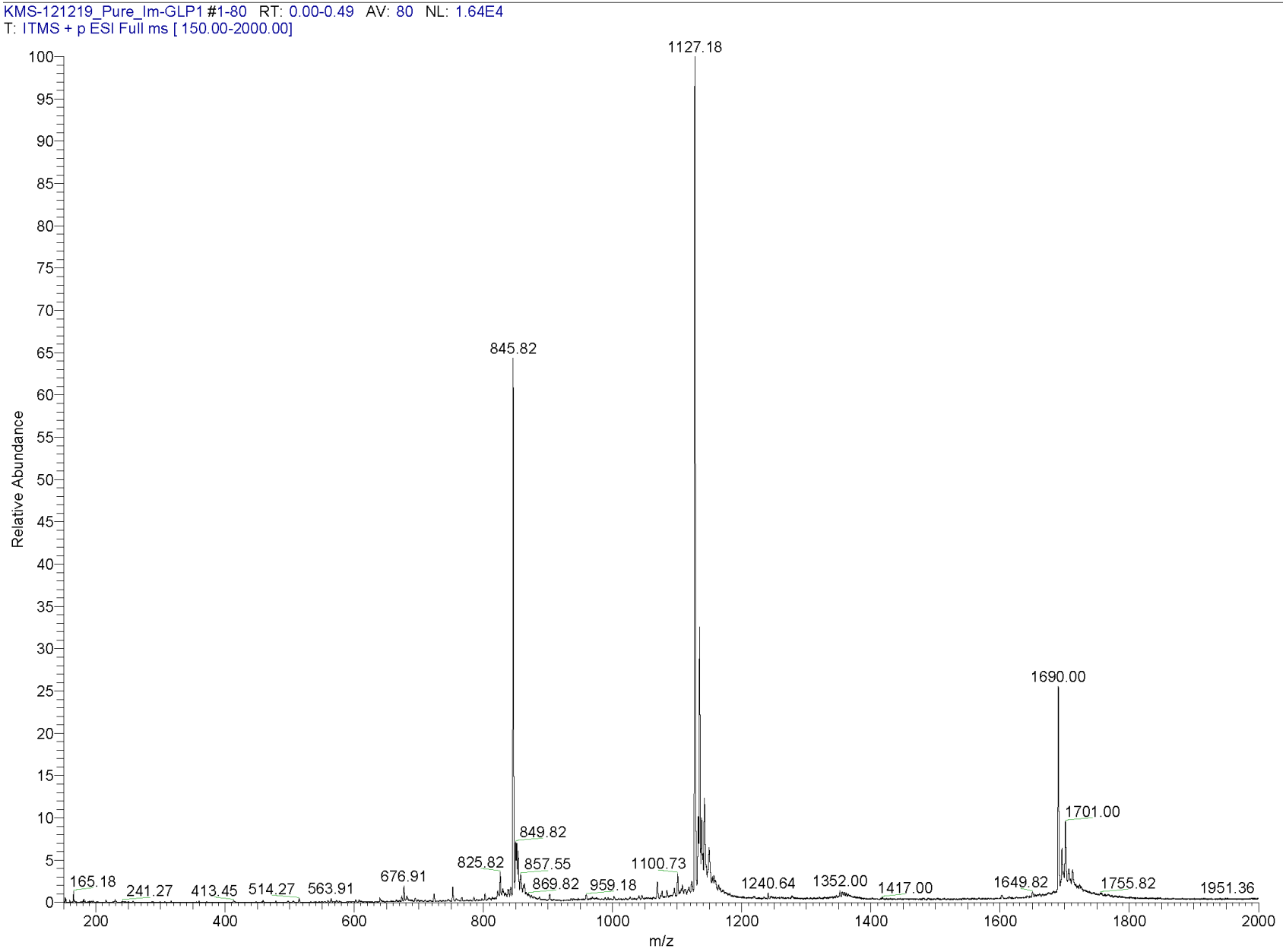
9-GLP1


KMS-021317-4-hydroxyl-benzyl-GLP1_Venkat_pure #1-87 RT: 0.00-0.49 AV: 87 NL: 1.31E5
T: ITMS + p ESI Full ms [150.00-2000.00]
Relative Abundance
m/z
1135.64
852.00
825.45
857.64
1100.27
863.00
958.64
749.36
613.36
231.09
359.36
431.00
505.36
1349.18
1452.73
1649.27
1702.82
1713.82
1724.82
1735.82
1746.73
1786.82
1893.55

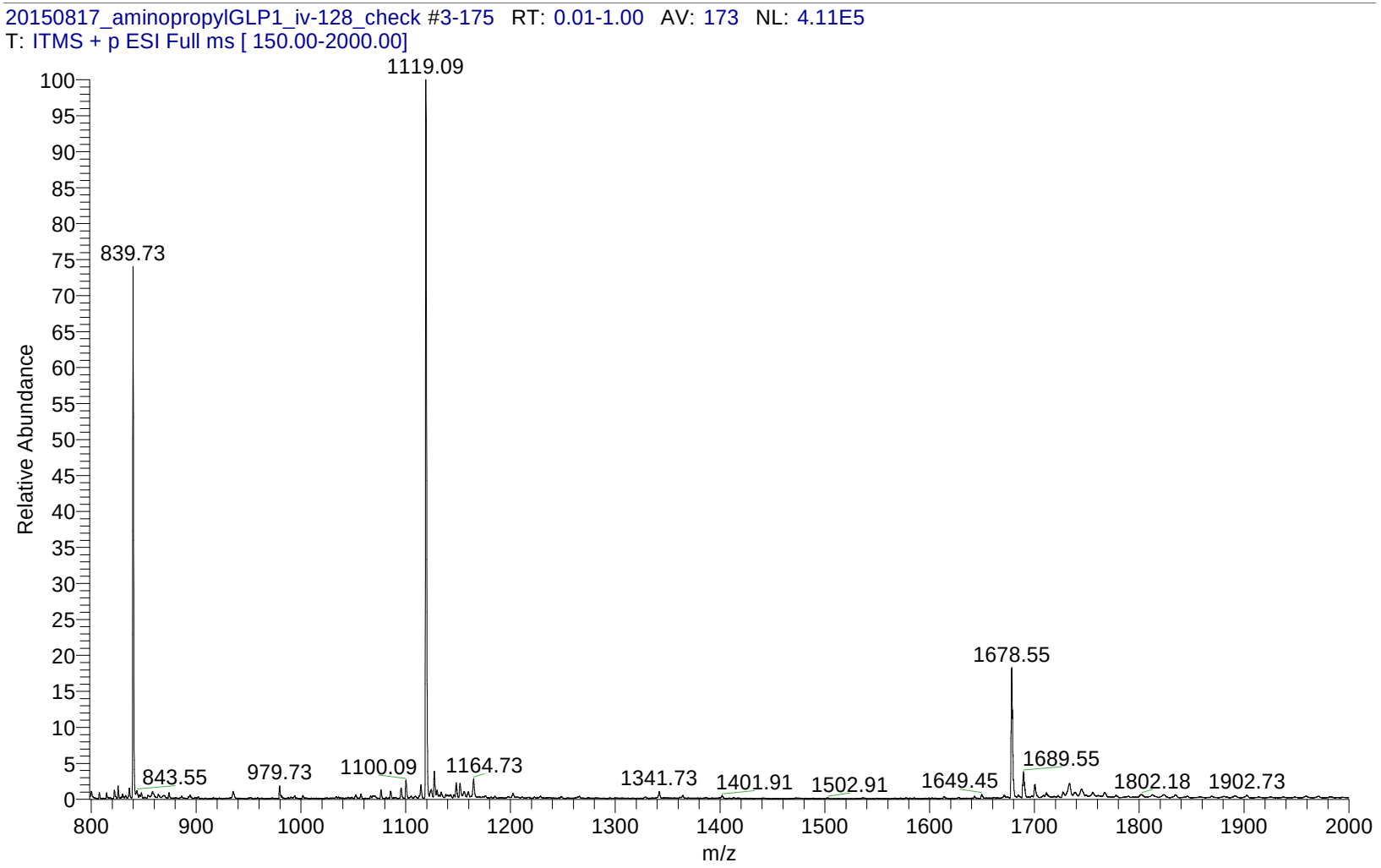
10-GLP1


20150711_BenzylGLP1_150711150742 #11 RT: 0.06 AV: 1 NL: 1.13E6
T: ITMS + p ESI Full ms [150.00-2000.00]
Relative Abundance
m/z
1130.09
848.00
1694.55
707.91
837.27
987.64
853.82
1706.00
1717.00
200.09
271.18
458.45
586.45
678.55
1255.27
1355.55
1480.91
1581.36
1837.27
1967.27

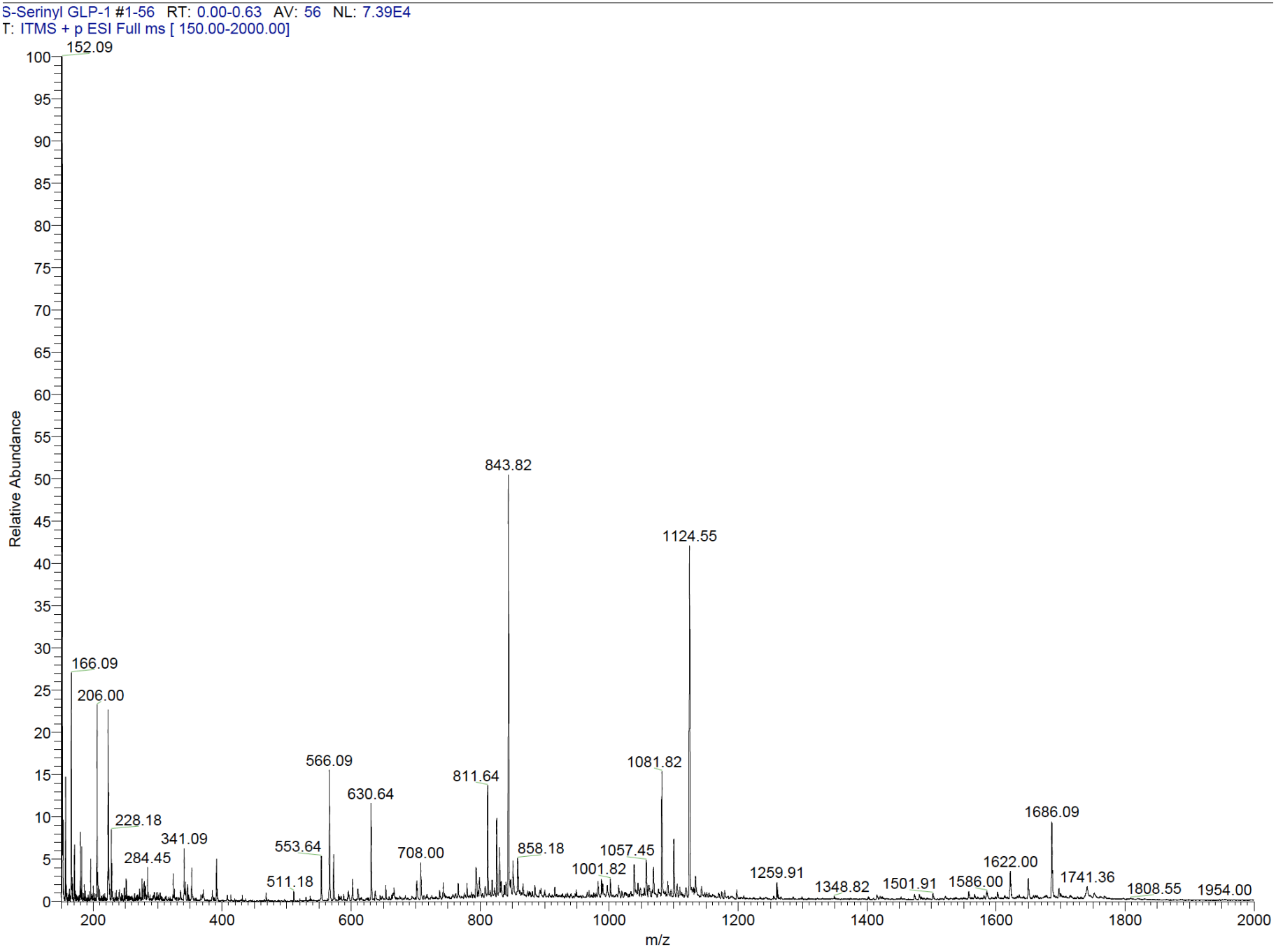
11-GLP1


KMS-121219_Pure_Im-GLP1 #1-80 RT: 0.00-0.49 AV: 80 NL: 1.64E4
T: ITMS + p ESI Full ms [150.00-2000.00]
Relative Abundance
m/z
1127.18
845.82
1690.00
1701.00
849.82
825.82
857.55
1100.73
676.91
869.82
959.18
1240.64
1352.00
1417.00
1649.82
1755.82
1951.36
165.18
241.27
413.45
514.27
563.91

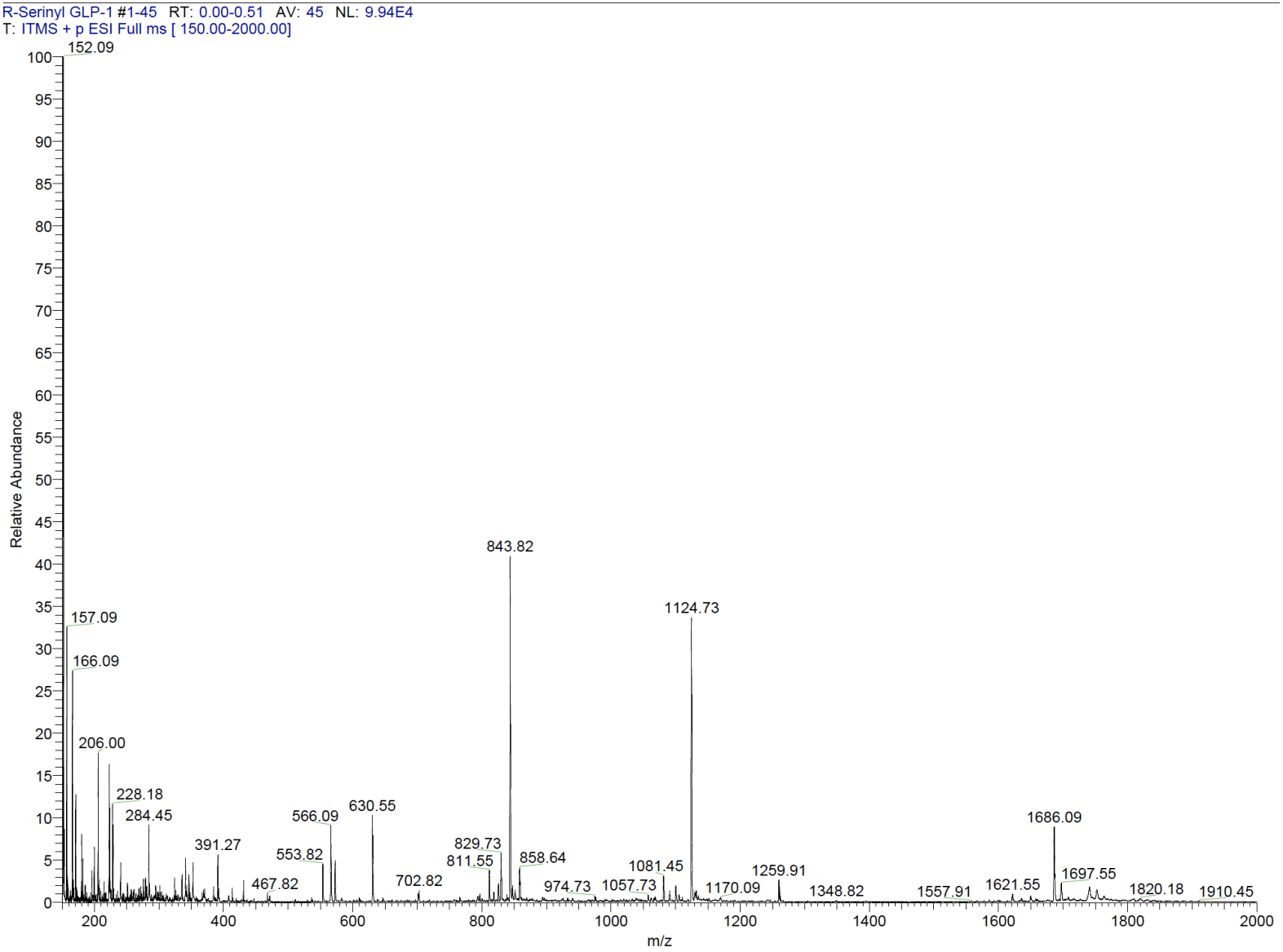
12-GLP1


20150817_aminopropylGLP1_iv-128_check #3-175 RT: 0.01-1.00 AV: 173 NL: 4.11E5
T: ITMS + p ESI Full ms [150.00-2000.00]
Relative Abundance
m/z
1119.09
839.73
1678.55
1689.55
843.55
979.73
1100.09
1164.73
1341.73
1401.91
1502.91
1649.45
1802.18
1902.73

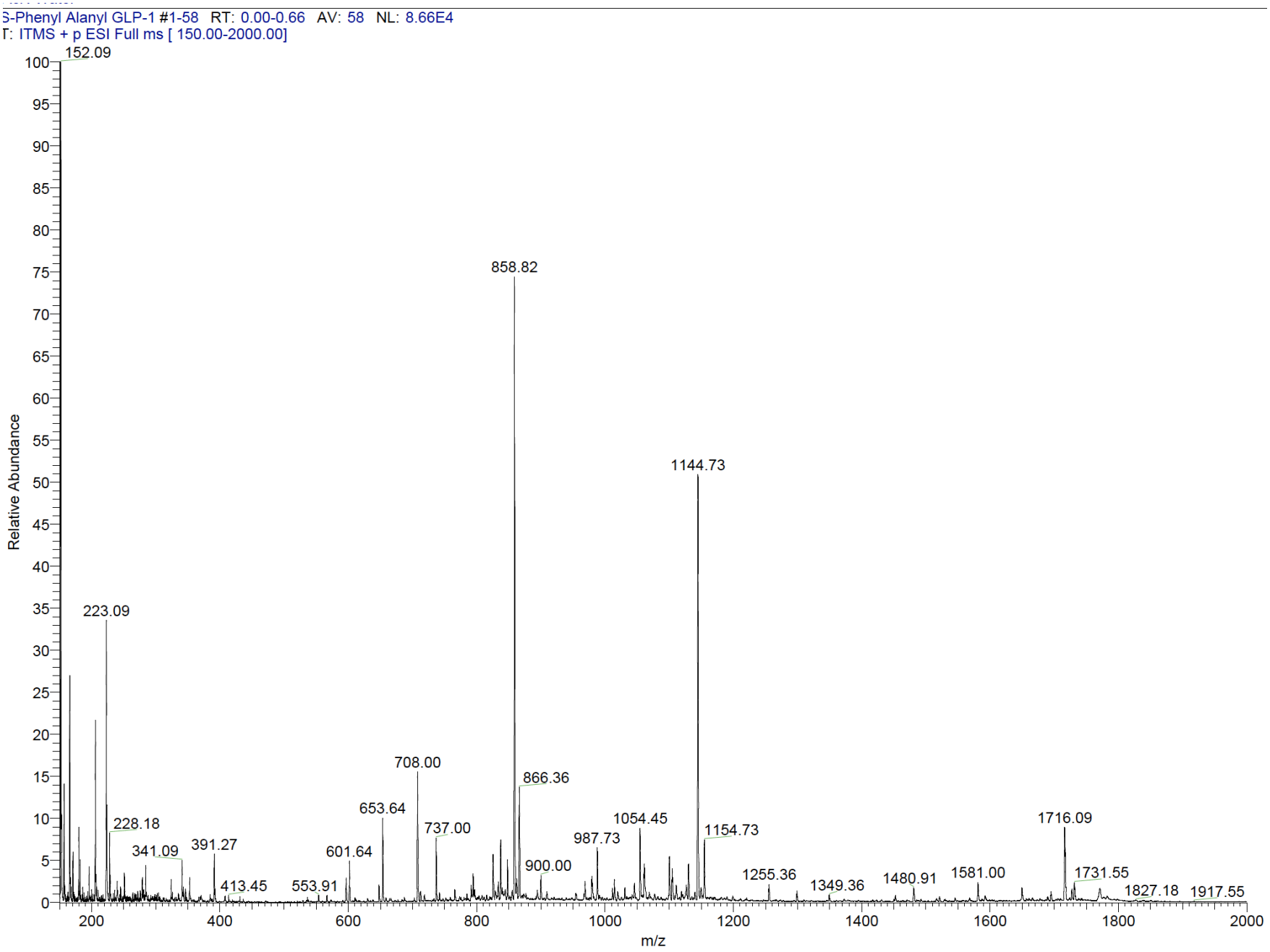
13-GLP1


S-Serinyl GLP-1 #1-56 RT: 0.00-0.63 AV: 56 NL: 7.39E4
T: ITMS + p ESI Full ms [150.00-2000.00]
Relative Abundance
m/z
152.09
843.82
1124.55
166.09
206.00
566.09
1081.82
811.64
630.64
228.18
341.09
284.45
553.64
708.00
858.18
1057.45
1001.82
1686.09
1622.00
1259.91
1741.36
1348.82
1501.91
1586.00
1808.55
1954.00
511.18

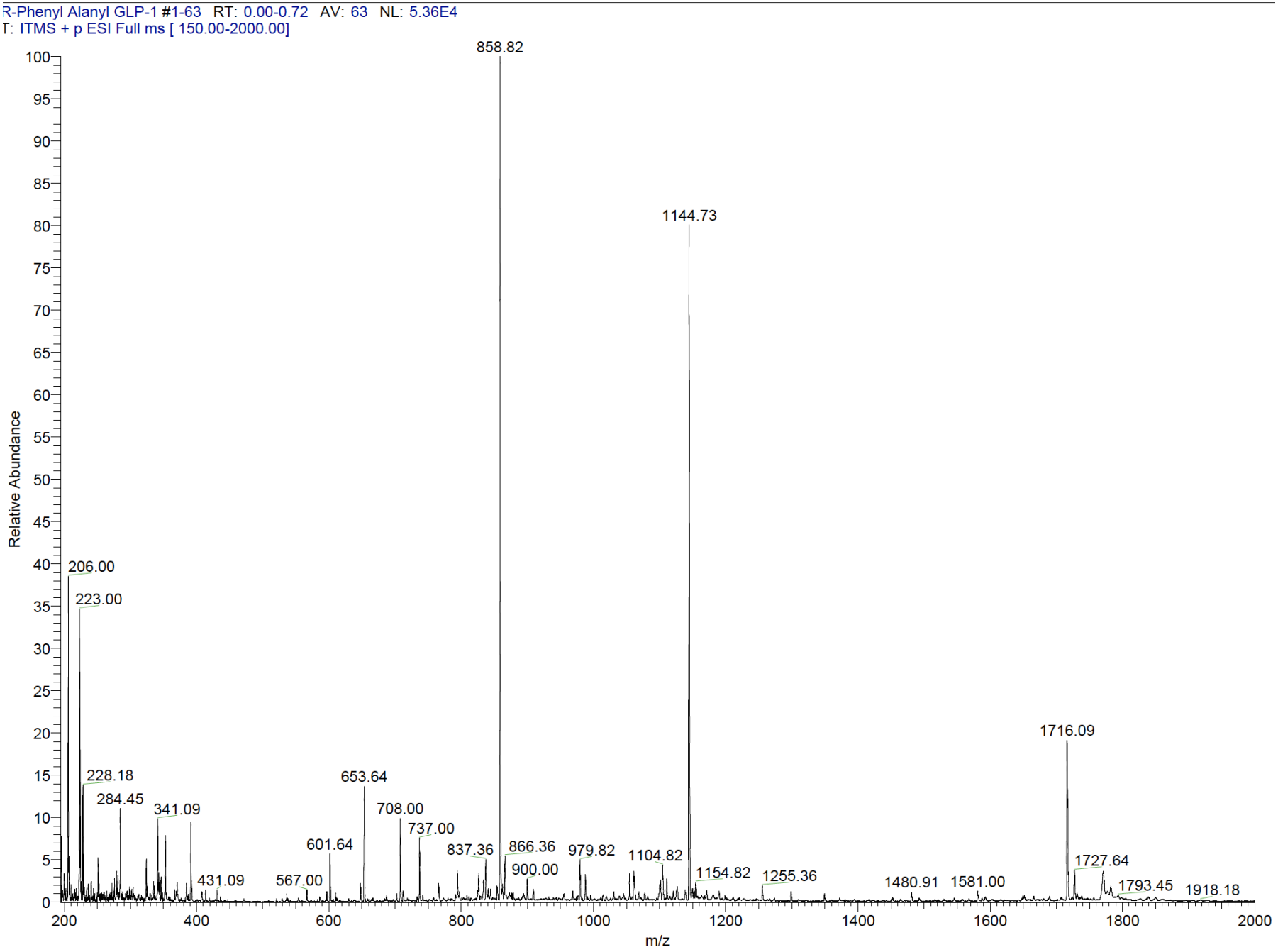
14-GLP1


R-Serinyl GLP-1 #1-45 RT: 0.00-0.51 AV: 45 NL: 9.94E4
T: ITMS + p ESI Full ms [150.00-2000.00]
Relative Abundance
m/z
152.09
157.09
166.09
206.00
228.18
284.45
391.27
467.82
553.82
566.09
630.55
702.82
811.55
829.73
843.82
858.64
974.73
1057.73
1081.45
1124.73
1170.09
1259.91
1348.82
1557.91
1621.55
1686.09
1697.55
1820.18
1910.45

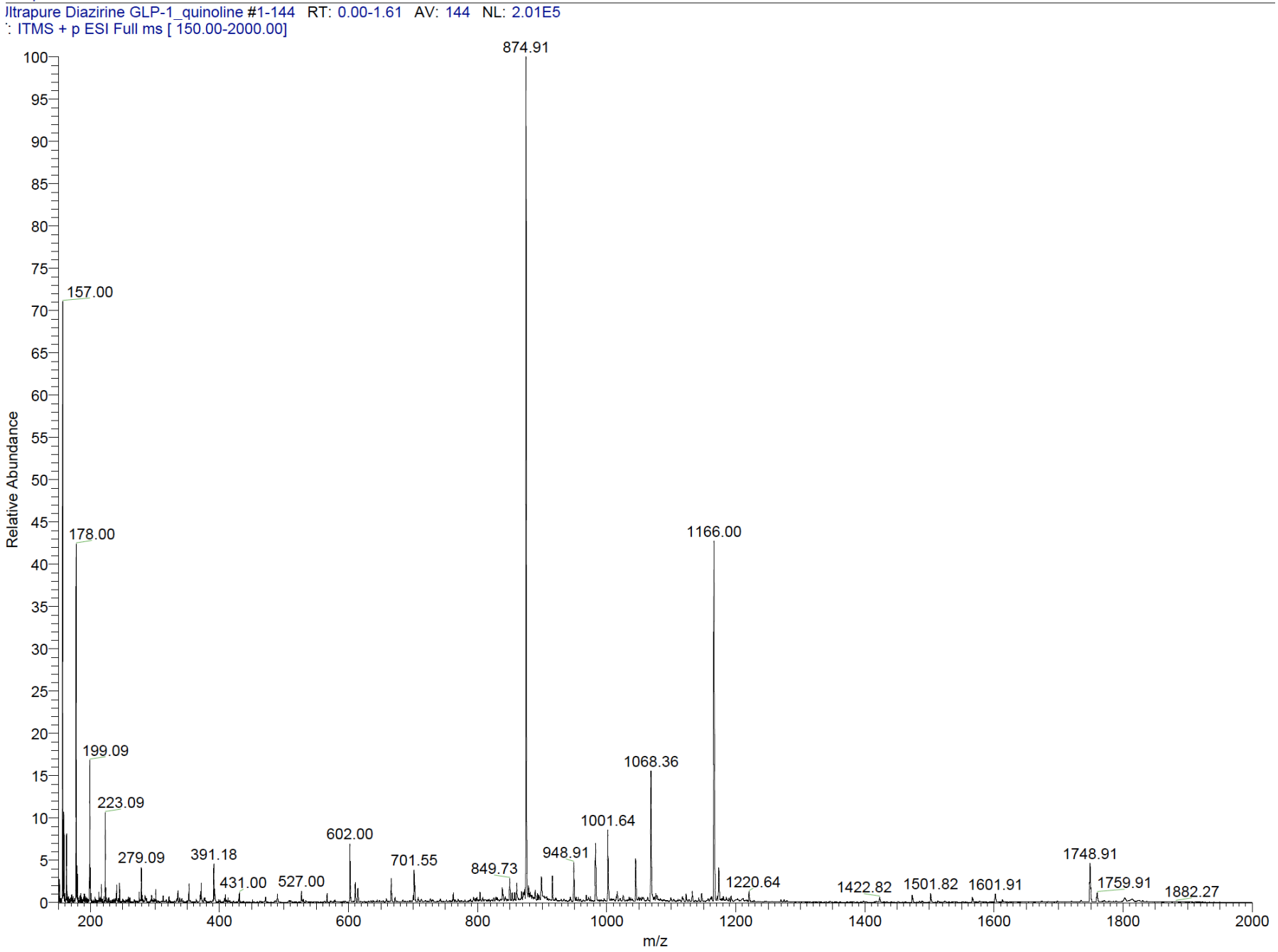
15-GLP1


S-Phenyl Alanyl GLP-1 #1-58 RT: 0.00-0.66 AV: 58 NL: 8.66E4
T: ITMS + p ESI Full ms [150.00-2000.00]
Relative Abundance
m/z
152.09
858.82
1144.73
223.09
708.00
866.36
653.64
737.00
228.18
1054.45
987.73
1154.73
1716.09
341.09
391.27
601.64
900.00
1255.36
1349.36
1480.91
1581.00
1731.55
413.45
553.91
1827.18
1917.55

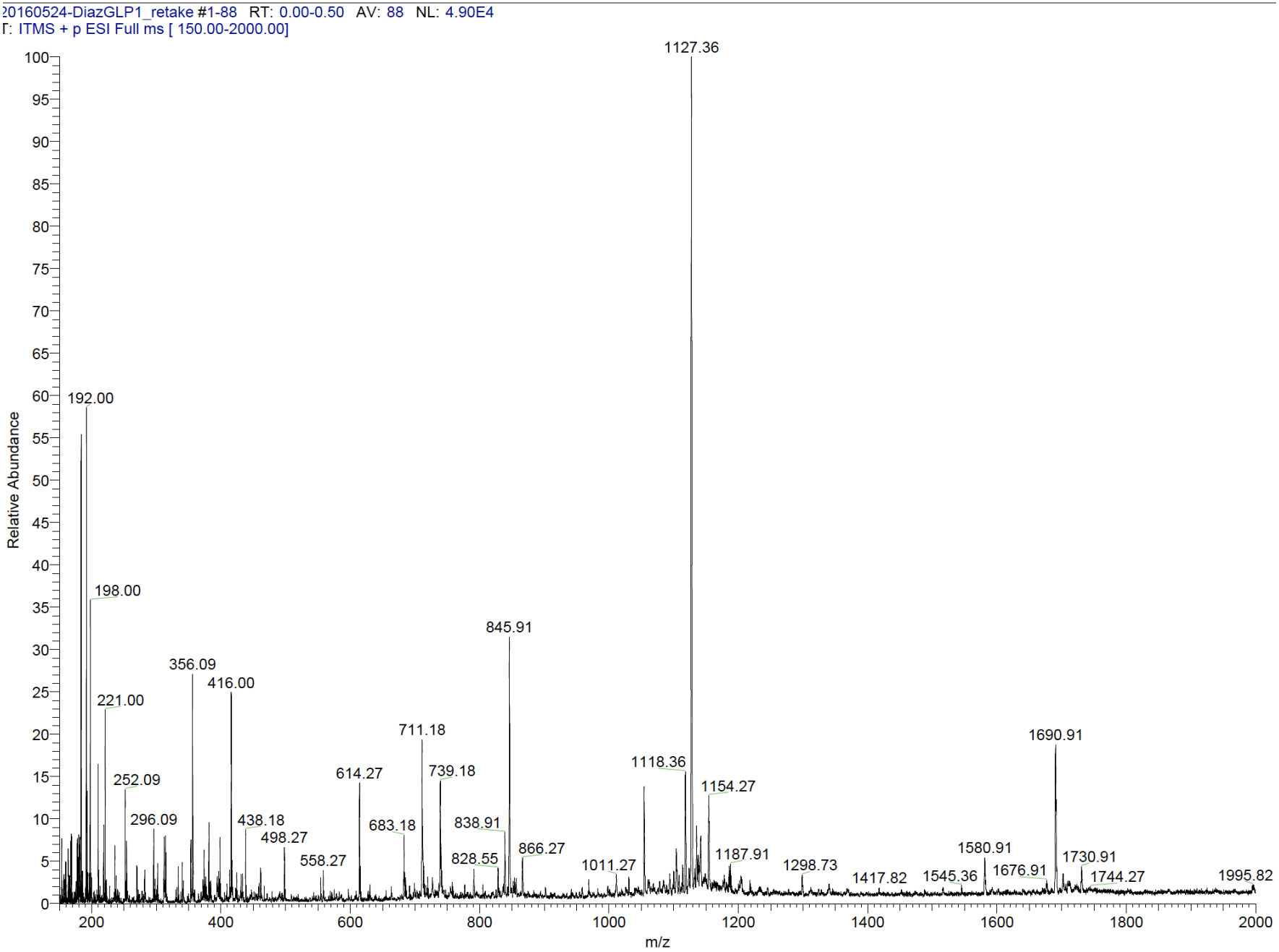
16-GLP1


R-Phenyl Alanyl GLP-1 #1-63 RT: 0.00-0.72 AV: 63 NL: 5.36E4
T: ITMS + p ESI Full ms [150.00-2000.00]
Relative Abundance
m/z
858.82
1144.73
1716.09
206.00
223.00
228.18
284.45
341.09
431.09
567.00
601.64
653.64
708.00
737.00
837.36
866.36
900.00
979.82
1104.82
1154.82
1255.36
1480.91
1581.00
1727.64
1793.45
1918.18

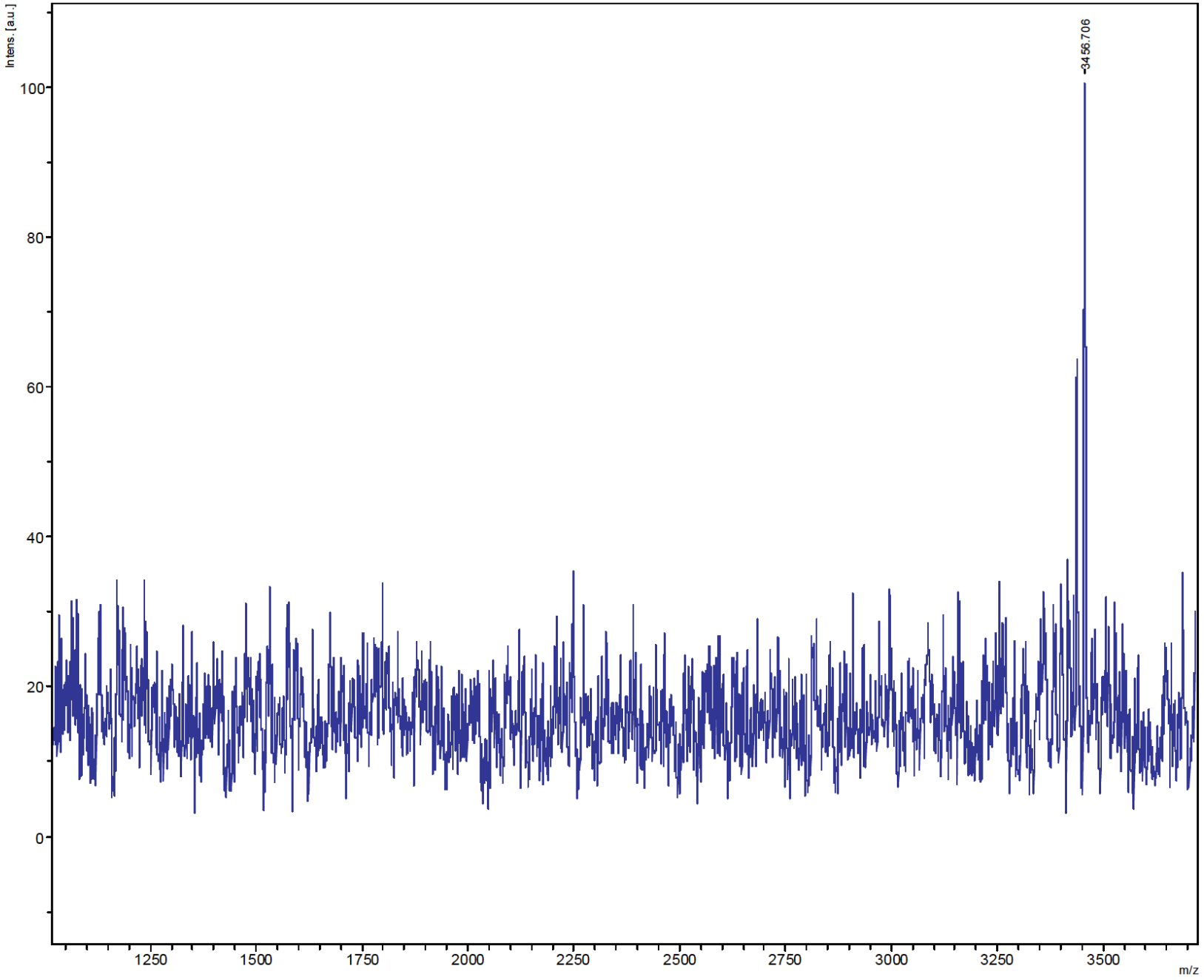
17-GLP1


Ultrapure Diazirine GLP-1_quinoline #1-144 RT: 0.00-1.61 AV: 144 NL: 2.01E5
: ITMS + p ESI Full ms [150.00-2000.00]
Relative Abundance
m/z
874.91
157.00
178.00
1166.00
199.09
1068.36
223.09
1001.64
602.00
279.09
391.18
701.55
948.91
849.73
431.00
527.00
1220.64
1748.91
1422.82
1501.82
1601.91
1759.91
1882.27

18-GLP1


20160524-DiazGLP1_retake #1-88 RT: 0.00-0.50 AV: 88 NL: 4.90E4
T: ITMS + p ESI Full ms [150.00-2000.00]
Relative Abundance
m/z
1127.36
192.00
198.00
845.91
356.09
416.00
221.00
711.18
739.18
614.27
1118.36
1154.27
1690.91
252.09
296.09
438.18
498.27
683.18
838.91
866.27
558.27
828.55
1011.27
1187.91
1298.73
1580.91
1730.91
1676.91
1744.27
1417.82
1545.36
1995.82

19-GLP1


Intens. [a.u.]
100
80
60
40
20
0
3456.706
1250
1500
1750
2000
2250
2500
2750
3000
3250
3500
m/z